Journal of the Endocrine Society, 2021, Vol. 5, No. 4, 1–16
doi:10.1210/jendso/bvab023
Research Article

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IGSF1 Does Not Regulate Spermatogenesis or Modify FSH Synthesis in Response to Inhibins or Activins

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Abbreviations: ACVR (1B/2A/2B), activin (type I/type II) receptor; ALK4, activin receptor-like kinase 4, also known as ACVR1B; AMH, anti-Müllerian hormone; ANOVA, analysis of variance; AUMC, Amsterdam University Medical Centers; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle-stimulating hormone; FT4, free thyroxine; LH, luteinizing hormone; LT4, levothyroxine; qPCR, quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SHBG, sex hormone–binding globulin; TSH, thyrotropin (thyroid-stimulating hormone); WHO, World Health Organization.

Received: 6 January 2021; Editorial Decision: 10 February 2021; First Published Online: 20 February 2021; Corrected and Typeset: 23 March 2021.

Abstract

Loss-of-function mutations in the X-linked immunoglobulin superfamily, member 1 (*IGSF1*) gene result in central hypothyroidism, often associated with macroorchidism. Testicular enlargement in these patients might be caused by increases in follicle-stimulating hormone (FSH) levels, as IGSF1 has been proposed to function as an inhibin B receptor or as an inhibitor of activin type I receptor (ALK4) activity in pituitary gonadotrope cells. If true, loss of IGSF1 should lead to reduced inhibin B action or disinhibition of activin signaling, thereby increasing FSH synthesis. Here, we show that FSH levels and sperm counts are normal in male *Igsf1* knockout mice, although testis size is mildly increased. Sperm parameters are also normal in men with IGSF1 deficiency, although their FSH

ISSN 2472-1972

levels may trend higher and their testes are enlarged. Inhibin B retains the ability to suppress FSH synthesis in pituitaries of *Igsf1*-knockout mice and IGSF1 does not interact with ALK4 or alter activin A/ALK4 stimulation of FSHβ (*Fshb/FSHB*) subunit transcription or expression. In light of these results, it is unlikely that macroorchidism in IGSF1 deficiency derives from alterations in spermatogenesis or inhibin/activin regulation of FSH.

Key Words: IGSF1, FSH, macroorchidism, pituitary

Mutations in the X-linked immunoglobulin superfamily, member 1 gene (*IGSF1*) are the most common genetic cause of congenital central hypothyroidism [1, 2]. Males with IGSF1 deficiency also exhibit prolactin and growth hormone dysregulation, disharmonious pubertal development, and macroorchidism [1-4]. Consistent with these phenotypes, IGSF1 is expressed in pituitary thyrotropes, lactotropes, and somatotropes in mice and rats, as well as in the testes of humans and rats [3, 5]. Macroorchidism is defined as testicular volumes > 2 SD above the mean in children, and as volumes > 30 mL by Prader orchidometer or > 18.3 mL by ultrasound in adults [6]. In IGSF1 deficiency, macroorchidism usually emerges during puberty with a normally or early timed, but somewhat prolonged, testicular volume increase, with testosterone production lagging behind. Almost 90% of adult male patients with IGSF1 deficiency develop overt macroorchidism, with sonographic testicular volumes of up to 70 mL [1, 3].

The etiology of macroorchidism in IGSF1 deficiency is unresolved, at least in part because the cellular functions of IGSF1 have not been resolved [2]. Potential causes include hypothyroidism and elevated follicle-stimulating hormone (FSH), both of which influence proliferation of testicular Sertoli cells during postnatal development [7, 8]. However, many individuals with IGSF1 deficiency have been treated with levothyroxine since infancy and, nevertheless, developed testicular enlargement [1, 9]. Therefore, hypothyroidism is unlikely to explain macroorchidism in these cases, although it is unknown whether thyroid hormone action in the testes of these patients is normal.

Men with FSH-hypersecreting pituitary adenomas have enlarged testes, consistent with the idea that elevated FSH might drive macroorchidism in IGSF1 deficiency [10]. Although basal FSH levels in IGSF1 deficient patients are within the normal range, they are somewhat elevated relative to luteinizing hormone (LH) [1]. In 4 out of 6 patients who underwent 24-hour frequent blood sampling, total FSH secretion was increased, but testicular size was not correlated with FSH levels [1, 11]. In one patient with a whole-gene deletion of *IGSF1*, macroorchidism was attributed to elevated FSH levels during "mini-puberty," although no cause-effect relationship was established [12]. Interestingly, at age 14 years, this patient had normal FSH levels despite having elevated inhibin B levels, which the authors attributed to pituitary inhibin B insensitivity because IGSF1 was initially proposed to function as an inhibin B co-receptor in pituitary gonadotrope cells [13, 14].

Inhibins A and B are gonadal ligands of the transforming growth factor β superfamily that act in an endocrine manner to selectively inhibit FSH production [15-17]. Although the ovary produces both inhibin A and B, only inhibin B is secreted by Sertoli cells in males of most mammalian species, including humans and rodents [16, 17]. Therefore, if IGSF1 functions as an inhibin B co-receptor, its loss should lead to impaired inhibin action, elevated FSH, enhanced Sertoli cell proliferation during postnatal development and, as a result, macroorchidism. However, the role of IGSF1 in inhibin B action is disputed. For example, inhibin A and B do not bind IGSF1 [18], and FSH production is normal in *Igsf1* knockout mice [19, 20]. In the most extensive analysis of IGSF1 patients, inhibin B levels correlated with FSH ($P = 0.06$) but not with ultrasonographic testicular volumes ($P = 0.67$) [1]. In addition, the IGSF1 protein was not detected in gonadotropes of mice or rats [3, 5]. Nevertheless, more recently, IGSF1 protein and *Igsf1* mRNA were reportedly expressed in adult rat gonadotropes when assessed with a different antibody and cell fractionation approach [12].

A novel mechanism was also proposed in which IGSF1 might regulate FSH production by inhibiting the activity of the activin type I receptor, ACVR1B (also known as activin receptor-like kinase 4 or ALK4) (see schematic in Fig. 5B of reference [12]). Activins are transforming growth factor β ligands [21, 22] that stimulate synthesis of FSH by promoting transcription of its beta subunit gene (*Fshb*) [23-25]. The ligand binds the activin type II receptors, ACVR2A and ACVR2B [26-29], which then *trans*-phosphorylate the activin type I receptors ALK4 and ALK7 [30]. ALK4/7 phosphorylate the intracellular signaling protein SMAD3 (homolog of *Drosophila* mothers against decapentaplegic 3), which complexes with SMAD4 in the cytoplasm, and translocates to the nucleus. SMAD3/4 then partner with forkhead box L2 (FOXL2), bind the *Fshb* proximal promoter, and stimulate *Fshb* transcription [25, 31-37]. According to the recently proposed model, IGSF1 interacts with and inhibits

ALK4 activity in gonadotropes [12]. As a result, in the absence of IGSF1, ALK4 activity would be increased (through disinhibition), leading to enhanced FSH production. An appealing aspect of this model is that it could explain modulation of FSH levels even if IGSF1 does not regulate inhibin action; however, it has yet to be independently validated.

In the present study, we investigated sperm parameters in IGSF1-deficient men and male mice to determine whether macroorchidism is associated with alterations in spermatogenesis. This would be predicted if FSH-driven increases in Sertoli cell numbers underlie testicular enlargement. We then tested whether IGSF1 modulates inhibin action or activin type I receptor (ALK4) activity to regulate FSH synthesis.

Methods

Human Participants

All known Dutch male patients from 18 to 80 years of age with IGSF1 deficiency (n = 15) were invited to participate in the study. Inclusion criteria were age ≥18 years, and the capability to donate semen. Exclusion criteria were congenital abnormalities or use of medication known to affect semen quality. All patients visited the hospital twice for semen collection and blood withdrawal. Clinical data regarding the patients' diagnosis and past and current treatment were retrieved from their medical records and earlier published information [1, 6]. Testicular volume was measured by ultrasound and compared with reference data as described previously [6]. The ethics committee of the Amsterdam University Medical Centers (AUMC) approved the study protocol. The study was conducted in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

Semen Analysis in Patients

All patients participated in this study between April and October 2016. Patients were instructed to refrain from ejaculation for a minimum of 2, and a maximum of 7 days, and to keep the number of days of abstinence similar before each visit. An interval between visits of at least 3 days was maintained to allow for the minimum of 2 days of abstinence. The duration of abstinence was recorded when the first semen sample was collected. Samples were collected at the AUMC by donation into a sterile container and were analyzed within 1 hour after ejaculation. After liquefaction at 37 °C, semen volume and pH were determined. The concentration of spermatozoa and the motility of at least 100 spermatozoa were assessed. Motility was scored as progressive, slow, or immotile. As it is contentious whether assessment of morphology increases the value of semen analysis, morphology is not routinely assessed in the AUMC. All parameters were classified according to the 2010 World Health Organization (WHO) criteria [38].

Endocrine Evaluation in Patients

For practical reasons, blood was collected on both study days between 11:00 and 15:00 hours rather than exclusively in the early morning. In both samples, concentrations of reproductive and thyroid hormones were measured. Reproductive hormones were compared with general population reference intervals and earlier reported values in male patients with IGSF1 deficiency [1]. Plasma free thyroxine (FT4) concentrations were measured by fluoroimmunoassay using the Delfia 1232 Fluorometer (Wallac, Turku, Finland). Plasma thyroid-stimulating hormone (TSH), FSH, LH, and anti-Müllerian hormone (AMH) were measured by electrochemiluminescence assay using the Roche cobas e601 or e602 (Roche Diagnostics, Mannheim, Germany). Plasma testosterone and androstenedione were measured by an in-house method using the Acquity ultra-performance liquid chromatography-tandem mass spectrometry system (Waters, Milford, Mam USA). Serum sex hormone–binding globulin (SHBG) was measured with immunoluminometric assay using the Architect iSR2000 (Abbott Laboratories, Diagnostics Division, Illinois USA). Plasma dehydroepiandrosterone sulfate (DHEAS) concentrations were determined by radioimmunoassay (Beckman Coulter, Brea, CA, USA) using a COBRA II Gamma-Counter (Packard Bioscience, Groningen, Netherlands). Inhibin B was measured by enzymatic immunoassay (Beckman Coulter) using the VICTOR Multilabel Plate Reader (Perkin-Elmer, Rodgau, Germany).

Mice

Igsf1$^{\Delta 312}$ mice were previously described (*Igsf1*^{em1Djb}; MGI: 5779502) [20]. *Igsf1*$^{\Delta 312/+}$ females were crossed with *Igsf1*$^{+/y}$ (wild-type) males, and *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ (knockout) males generated from this cross were compared. Animals were housed on a 12:12-hour light/dark cycle and given ad libitum access to food and water. All animal work was conducted in accordance with federal and institutional guidelines and with the approval of the Goodman Cancer Centre Facility Animal Care Committee at McGill University (protocol no. 5204).

Blood Collection and Hormone Analyses in Mice

Ten- to 12-week-old male *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ littermates were anesthetized with isoflurane and euthanized by cervical

dislocation. Blood was collected by cardiac puncture, allowed to coagulate at room temperature for 30 minutes and spun down for 10 minutes at 800*g*. The serum was collected and stored at –20 °C. Serum LH was assessed using an in-house ELISA as previously described (detection range: 0.117 to 30 ng/mL) [39]. Serum FSH was measured with a Milliplex MAP mouse pituitary magnetic bead panel (MilliporeSigma, MPTMAG-49K [custom-made for FSH only], Oakville, Ontario, Canada) following the manufacturer's instructions (minimal detection limit: 9.5 pg/mL). Serum inhibin B was measured by ELISA (Ansh; performed at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia; minimal detection limit: 8 pg/mL).

Organ Collection From Mice and Rats

Pituitary glands, testes, epididymides, and seminal vesicles were dissected from 10- to 12-week-old male *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ mice. Pituitaries were also extracted from 3 lactating adult female Sprague Dawley rats, 1-3 days postpartum. Pituitary glands (rats and mice) and left testes (mice) were snap-frozen in liquid nitrogen and stored at –80 °C. The right testis, cauda epididymis, and the seminal vesicle were weighed on an analytical balance. The right caudal epididymis was snap-frozen, and later used for the sperm count as described previously [35, 40]. The right testis was immersed in Bouin's Fixative Solution (1120-16, Ricca Chemical Company, Arlington, TX) overnight at room temperature, followed by a 24-hour incubation in 100% ethanol. Testes were stored in 70% ethanol. Two fixed testes from each genotype were paraffin-embedded, sectioned, and hematoxylin/eosin stained at the McGill Centre for Bone and Periodontal Research. Images were acquired with a Leica Microsystems DFC310 FC1.4-megapixel digital color camera on a Leica Microsystems DM1000 light-emitting diode microscope.

Computer-Assisted Sperm Analysis

Left caudal epididymides were dissected, placed in warm (37 °C) M199 media with Hank's salt (M7653, MilliporeSigma), supplemented with 0.5% bovine serum albumin (w/v) (A4378, MilliporeSigma) and cut 7 times. Sperm cells were allowed to disperse in media for 5 minutes at 37 °C and then diluted 4 times. Computer-assisted semen analysis was performed using a TOX IVOS automated semen analyzer (Hamilton Thorne, Beverly, MA).

DNA Constructs

Murine FLAG-SMAD4 and rat ALK4-HA (both in pcDNA3.0) were provided by Dr. Teresa Woodruff (Northwestern University, Chicago, Illinois). Rat ALK4-myc was made in-house by subcloning the rat ALK4 cDNA from ALK4-HA into pcDNA4 (V86320, Invitrogen, Waltham, MA), which added C-terminal myc and His tags. Rat ALK4-T206D-HA (ALK4TD) and untagged murine SMAD3 [41], murine IGSF1 and IGSF1-HA [20], human myc-IGSF1-HA [3], –1195/+1 *Fshb*-luc and –1028/+7 C-165T *FSHB*-luc reporters [31], and the $CAGA_{12}$-luc reporter [42] were all previously described.

Murine Pituitary Cell Cultures

Primary pituitary cultures were prepared as previously described [43]. Pituitaries were collected and dispersed from 8- to 12-week-old *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ males. The cells were seeded (at 300 000 cells/well) in 48-well plates and cultured in M199 medium (M7653, MilliporeSigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (10438026, Wisent Inc., St-Bruno, QC, Canada). The following day, cells were treated for 24 hours with recombinant inhibin A (0.01, 0.1, or 1 nM; 624-IN/CF, R&D systems, Minneapolis, MN) or inhibin B (0.01, 0.1, or 1 nM; 677-IB/CF, R&D Systems) diluted in M199 medium containing 2% FBS. Following treatment, media was removed, and RNA was extracted using the Total RNA Mini Kit (FA32808-PS, Geneaid, New Taipei City, Taiwan) following the manufacturer's guidelines.

Immunoprecipitation, Deglycosylation, and Immunoblotting

HEK293 cells (provided by Dr. Terry Hébert, McGill University, Montréal) and LβT2 cells (provided by Dr. Pamela Mellon, University of California, San Diego, CA) [44] were cultured in DMEM (4.5 g/L glucose, with L-glutamine and sodium pyruvate) containing either 5% or 10% (v/v) FBS, respectively. For protein extraction, cells were seeded in 6-well plates; HEK293 cells at a density of 600 000 cells/well and LβT2 cells at a density of 1 800 000 cells/well. The following day, HEK293 cells were transfected with a total of 2 µg of pcDNA3.0, murine IGSF1, murine IGSF1-HA, rat ALK4-myc, and/or rat ALK4-HA plasmid(s) per well using polyethylenimine at a concentration of 1:3 total DNA to polyethylenimine. LβT2 cells were transfected with 500 ng ALK4TD and 0, 125, 250, or 500 ng human myc-IGSF1-HA balanced with pcDNA3.0 for a total of 1 µg/well using 2.4 µl each of P3000 Reagent and Lipofectamine 3000 Transfection Reagent per well (L3000015, ThermoFisher Scientific, Waltham, MA). At 24 hours post-transfection, total protein lysates were extracted as previously described [20]. For immunoprecipitation assays, cells were lysed in buffer

(50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) at 24 hours post-transfection and total protein lysates were incubated with 4 µg murine monoclonal anti-HA (H9658, Sigma-Aldrich) on a rotator overnight at 4 °C. The following day, 15 µL protein A/G agarose beads (20421, ThermoFisher Scientific) were added to the mix, and incubated for 3 hours at 4 °C. The agarose beads were spun down at 3600 g for 30 seconds at 4 °C, and supernatant removed. Precipitated proteins were eluted by heating in Laemmli buffer at 75 °C for 15 minutes. For lysates from tissues, pituitary glands were processed as previously described [20].

Protein concentrations were measured using the Pierce BCA protein assay kit (23227, ThermoFisher Scientific) following the manufacturer's instructions. Where indicated, 20 µg of each protein lysate were denatured at 72 °C for 15 minutes, cooled on ice for 10 minutes and then deglycosylated with 500 U PNGaseF (P0704S, New England BioLabs) or left untreated for 2 hours at 37 °C prior to being resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In all other cases, 20 µg of protein lysate were denatured at 70 °C for 15 minutes and immunoblotted as previously described [45]. The monoclonal mouse anti-c-Myc (1:10 000, M5546) [46], anti-HA (1:40 000, H9658) [47] and anti-β-actin (1:10 000, A5441) [48] were from Sigma-Aldrich. The rabbit anti-IGSF1-CTD antibody (1:1000) was previously described [49, 50] and the commercial rabbit anti-IGSF1 (1:500, GTX112633) was from GeneTex (Irvine, CA) [51].

Reverse Transcription Quantitative Polymerase Chain Reaction

For induction of endogenous *Fshb*, LβT2 cells were seeded and transfected as described in the previous section. At 24 hours post-transfection, cells were serum starved for an additional 24 hours prior to RNA extraction. Total RNA was isolated with TRIzol reagent (15596026, Life Technologies, Carlsbad, CA) following the manufacturer's guidelines. RNA concentrations were determined by NanoDrop. For cDNA synthesis, 500 ng (LβT2 cells) or 100 ng (primary cells) of RNA were reverse transcribed as previously described [45]. The resulting cDNA was used for quantitative polymerase chain reaction (qPCR) analysis on a Corbett Rotorgene 600 instrument (Corbett Life Science, Sydney, Australia) using EvaGreen (ABMMmix-S, Diamed, Mississauga, ON, Canada) and primers listed in Table 1. The mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method. Gene expression was normalized to ribosomal protein L19 (*Rpl19*). All primers were validated for efficiency and specificity.

Promoter-Reporter Assays

Promoter-reporter assays were performed as previously described [45]. Briefly, cells were seeded in 48-well plates at a density of 50 000 cells/well (HEK293) or 150 000 cells/well (LβT2). Approximately 24 hours later, cells were transfected using either a 1:3 ratio of DNA and polyethylenimine (HEK293) or 0.3 µl/well each of P3000 Reagent and Lipofectamine 3000 Transfection Reagent (LβT2), 225ng/well of the indicated luciferase reporter, and a combination of expression vectors for IGSF1 (0-100 ng/well, as indicated in the figures), 10 ng/well ALK4TD, 4.15 ng/well FOXL2, 25 ng/well SMAD3, and/or 25 ng/well SMAD4, depending on the experiment. Where appropriate, the transfection was balanced with pcDNA3.0 to ensure the same amount of DNA per well. At 24 hours post-transfection, cells were serum starved for an additional 24 hours. After serum starvation, cells were either lysed or treated with 25 ng/mL activin A (or vehicle) 6 hours prior to lysis, as described previously [33]. Luciferase assays were performed using an Orion II microplate luminometer (Berthold Detection Systems, Oak Ridge, TN). All conditions were performed in triplicate wells in 4 independent experiments.

Analyses of Single-Cell RNA Sequencing Data

The t-distributed stochastic neighbor embedding (tSNE) plots were generated using previously published data from male mouse [52] and rat [53] pituitary single-cell RNA sequencing data using Loupe Browser 4.0 (10x Genomics, Pleasanton, CA).

Table 1. Genotyping and qPCR Primers

	Primer sequence	
Gene	**Forward**	**Reverse**
Igsf1$^{\Delta 312}$ genotyping	GGGTGACTGGTAAGGTTCTG	CAACAGGCCCTGTGGTATATC
Rpl19 qPCR	CGGGAATCCAAGAAGATTGA	TTCAGCTTGTGGATGTGCTC
Fshb qPCR	GTGCGGGCTACTGCTACACT	CAGGCAATCTTACGGTCTCG
Igsf1 qPCR	TGAGTTGGGTCAAGAGGATT	TGAGGAGTTACCAGGATAGAGGA

Statistical Analysis

Spearman's rank-order correlation was used to calculate the correlation coefficient between testicular volume and total sperm count in men. Effects of genotype on serum FSH, LH, and inhibin B, organ weights, and sperm concentration and motility in mice were assessed by unpaired *t*-tests with Welch's correction. Data in primary culture experiments were log transformed and analyzed by one-way analysis of variance (ANOVA), followed by Holm-Sidak correction. Each treatment group was compared with its own no-treatment or vehicle controls. Data in luciferase assays were log transformed and analyzed using two-way ANOVA followed by Dunnett's correction. Statistical analyses were performed using Prism 8, GraphPad software. $P < 0.05$ was considered statistically significant.

Results

Characteristics of IGSF1-Deficient Men

Five men with IGSF1 deficiency were included in the study, with a mean age of 27.2 years (Table 2). None had fathered a child or had a previous or current wish to do so. Three of the 5 patients were diagnosed with central hypothyroidism during infancy, after detection by the neonatal screening for congenital hypothyroidism. Another patient was diagnosed at age 7 years, after referral to a pediatric endocrinologist for growth failure. The fifth patient was diagnosed at age 25 years within the framework of a family study after a relative was diagnosed with IGSF1 deficiency. The first 4 patients were treated with levothyroxine (LT4, see Table 2). Patients 1, 2, and 4 were monitored in the AUMC for their hypothyroidism. Patient 3 attended the Leiden University Medical Center (LUMC) for his hypothyroidism. Patient 5 was diagnosed with a mutation in *IGSF1* in the LUMC but was not treated for hypothyroidism.

During the current study, 2 LT4-treated patients had FT4 concentrations within the reference interval in both blood samples, while the other 2 had one sample within the reference interval, and one sample outside the reference interval. TSH concentrations were <0.01 mU/L in all 4 LT4-treated patients, in both blood samples. The untreated patient had FT4 concentrations just above the lower limit of the reference interval, while his TSH concentrations were within the reference interval.

Patients had undergone extensive physical examinations previously, which was not repeated for this study. None had undergone testicular surgery, or experienced either testicular trauma or sexually transmitted infections capable of influencing semen parameters.

Overt macroorchidism was previously observed in all included patients, and median testicular size as measured by ultrasound was 25.0 ± 5.8 mL (+3.4 SDS) [6]. Ultrasound showed no testicular tissue abnormalities, including hydroceles or varicoceles, in any of the patients (Table 2).

Measurements of reproductive hormones compared with both general population reference intervals as well as earlier reported ranges in male IGSF1 deficiency patients are shown in Table 2. The FSH to LH ratio was high in all patients (average 2.1; range, 1.42-2.76) compared with described control groups consisting of proven fathers (0.7-1.2) and men with normozoospermia (0.65-1.05) [54]. The ratio in our patients was similar to that reported earlier for IGSF1 patients (2.2; interquartile range, 1.8-2.7) [1]. Testosterone concentrations were also within the reference interval, but 4 out of 5 patients had testosterone concentrations below the median, and all had SHBG concentrations below the median. One patient had elevated inhibin B concentrations, while 2 had increased levels of AMH. Both androstenedione and DHEAS concentrations were within the reference range. These results were similar to those observed in a larger group of patients, showing LH, androstenedione, and DHEAS concentrations in the lower half of the reference range [1].

Semen Characteristics Are Normal in IGSF1-Deficient Men

Two semen samples were analyzed from all 5 men. Semen characteristics are summarized in Table 3. One man had a semen volume below the WHO threshold of 1.5 mL in one sample. Sample pH, viscosity, and sperm aspect were normal in all men, and all had sperm counts above the WHO threshold of 15×10^6/mL. In one individual, sperm count was 291 and 295×10^6/mL on the first and second visit, respectively. While sperm counts >250×10^6/mL have been described as polyzoospermy, high values are clinically irrelevant and therefore there is no official upper limit [55]. There was no correlation between testicular volume and total sperm count ($P = 0.873$). While one man had progressive sperm motility below the WHO threshold of 40%, his motility was above the threshold of our age specific in-house reference intervals (>32%). Total motile count (calculated as volume × progressive motility × concentration/100) was above the in-house threshold of 10×10^6 in all. In these analyses, none of the included men were classified with astheno- or oligozoospermia according to the WHO criteria.

Table 2. Clinical and Endocrine Parameters in Males With IGSF1 Deficiency

Plasma levels (Reference interval)	**Patient 1**		**Patient 2**		**Patient 3**		**Patient 4**		**Patient 5**	
Mutation in *IGSF1* (nucleotide alteration; amino acid alteration)	Xq26.1q26.2 (130 386 267–130 512 002) × 0 (hg19)		c.2588C>T; p.Ser863Phe		c.2137_2163del; p.Ala713_Lys721del		c.2588C>T; p.Ser863Phe		c.2248delG; p.Glu750Lysfs[a]28	
Age, current (years)	26		31		25		27		27	
Adult right/left testicular volume by ultrasound (mL)	34[a]/25.5[a]		11.8/38[a]		30[a]/26[a]		25.5[a]/25.4[a]		16.1/18.6[a]	
Aspect testicular tissue right/left	Homogeneous/homogeneous		Epididymal cyst/homogeneous		Homogeneous/homogeneous		Homogeneous/homogeneous		Homogeneous/homogeneous	
Age at diagnosis central hypothyroidism	4 weeks		2 weeks		7 years		5 months		25 years	
Age at start of LT4 treatment	4 weeks		2 weeks-3 years; 28 years		7 years		5 months		N/A	
Body mass index	+2.8 SDS		+3.5 SDS		+1.8 SDS		+1.5 SDS		+1.5 SDS	
Visit	1	2	1	2	1	2	1	2	1	2
TSH (0.5-5.0 mIU/L)	<0.01[a]	<0.01[a]	<0.01[a]	0.02[a]	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]	1.70	1.80
FT4 (10-23 pmol/L)	21.5	20.1	14.5	8.6[a]	14.4	17.5	28.6[a]	20.7	10.6	10.4
FSH (0.1-15.0 U/L)	8.2	7.6	4.0	5.2	11.5	11.4	8.3	8.7	9.0	8.8
LH (0.1-15.0 U/L)	4.8	2.4	2.8	3.6	4.5	5.6	3.0	3.8	5.0	5.0
Testosterone (9.0-30.0 nmol/L)	12.4	10.6	13.3	13.8	13.8	13.8	25.1	20.0	14.1	11.1
SHBG (13-71 nmol/L)	13	13	18	17	23	20	38	41	20	21
Androstenedione (1.0-5.2 nmol/L)	2.3	1.3	1.2	1.7	2.2	2.1	3.9	3.0	2.6	1.9
DHEAS (0.8-17.0 µmol/L)	7.2	5.8	3.2	2.5	3.2	3.5	4.2	5.2	4.8	4.7
FAI (20.0-90.0)	95.4[a]	81.5	73.9	81.2	60.0	69.0	66.1	48.8	70.5	52.9
Inhibin B (150-400 ng/L)	472[a]	469[a]	238	256	175	168	235	219	255	282
AMH (1-12 µg/L)	38[a]	34[a]	5.7	5.7	12.9[a]	12.0	7.0	7.5	10.9	11.0

Age specific reference intervals: between parentheses.

Abbreviations: AMH, anti-Müllerian hormone; DHEA-S, dehydroepiandrosterone sulfate; FAI, free androgen index; FSH, follicle-stimulating hormone; FT4, free thyroxine; LH, luteinizing hormone; LT4, levothyroxine; N/A, not applicable; SHBG, sex hormone–binding globulin; TSH, thyroid-stimulating hormone.

[a] abnormal values

Table 3. Semen Analysis in Males With IGSF1 Deficiency

	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5	
Age (years)	26		31		25		27		27	
Visit	1	2	1	2	1	2	1	2	1	2
Abstinence (days)	4	3	5	6	3	3	2	2	2	2
pH (≥7.2)	7.7	7.5	7.5	7.5	7.5	7.5	8.0	7.7	7.5	7.5
MAR IgG (<50%)	0	0	0	0	0	0	x	0	0	0
Viscosity	Normal	Normal	Normal	Normal	Normal	Moderate	Normal	Normal	Normal	Normal
Aspect	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Volume (≥1.5 mL)	4.9	4.7	1.8	4.5	1.2[a]	2.3	3.8	4.4	1.8	1.5
Concentration (>15 × 10^6/mL)	78	70	115	144	295	291	75	65	96	189
Motility Progressive (>32%)	72	61	50	58	36	59	83	62	55	72
Non-progressive	7	7	17	16	23	12	3	10	15	16
Immotile	21	32	33	26	41	29	14	28	30	12
TMC (>10 × 10^6/mL)	275	201	104	376	127	395	237	177	95	204

Age specific in-house reference intervals: between parentheses.
Abbreviations: MAR, mixed antiglobulin reaction; TMC, total motile count (TMC calculated as volume × concentration × motility/100); x, missing data.
[a] abnormal values

Igsf1$^{\Delta 312/y}$ Mice Have Normal Reproductive Function

We previously reported that males of 2 different mouse strains with global (germline) deletions of *Igsf1* (*Igsf1*$^{\Delta ex1}$ and *Igsf1*$^{\Delta 312}$) are fertile with normal gonadotropin production [19, 20]. However, a systematic characterization of the reproductive function of *Igsf1*$^{\Delta 312/y}$ males was not described. Here, we found that serum concentrations of FSH (Fig. 1A), LH (Fig. 1B), and inhibin B (Fig. 1C) were comparable between *Igsf1*$^{+/y}$ (wild-type control) and *Igsf1*$^{\Delta 312/y}$ (knockout) littermates. Relative to wild-type, knockout mice had increased testis (Fig. 1D) but not seminal vesicle (Fig. 1E) or epididymal weights (Fig. 1F). Testis histology appeared normal (Fig. 1G), and caudal epididymal sperm concentrations (Fig. 1H) and progressive motility (Fig. 1I) did not differ between genotypes.

Cultured Pituitaries of *Igsf1*$^{\Delta 312/y}$ Males Respond Normally to Inhibins A and B

To directly assess whether IGSF1 regulates inhibin action in gonadotrope cells, we performed primary culture experiments using dispersed pituitary cells from both *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ mice. *Fshb* mRNA expression was similarly suppressed by inhibin A (Fig. 2A) and inhibin B (Fig. 2B) in cultures from both genotypes. *Lhb* and *Tshb* mRNA levels were also comparable between genotypes and were unaffected by inhibin treatment (data not shown), demonstrating the specificity of the inhibin effects. Reduced *Igsf1* mRNA expression levels in knockout cultures validated the genotypes of the mice (Fig. 2C-D).

IGSF1 Does Not Interact With ALK4 and Does Not Affect its Ability to Stimulate *Fshb* Expression

Although the above (and previously published) data appear to rule out a role for IGSF1 in inhibin action, it was possible that loss of IGSF1 could lead to enhanced activin signaling and, by extension, FSH production by gonadotropes [12]. However, the type I activin receptor, ALK4, interacted with itself (Fig. 3A, lane 6, second panel from the top), but not IGSF1 (lane 5, top panel) in co-immunoprecipitation experiments in heterologous cells. To examine whether IGSF1 regulates ALK4 activity, we used a robust assay in which a constitutively active form of ALK4 (ALK4TD) stimulates endogenous *Fshb* mRNA levels when overexpressed in the murine gonadotrope-like cell line, LβT2 [31, 41]. Neither ALK4TD-induced *Fshb* expression (Fig. 3B) nor ALK4TD protein levels (Fig. 3C) were affected by co-expression of IGSF1.

IGSF1 Does Not Inhibit Activin A Induction of *Fshb/FSHB* Promoter-Reporters in LβT2 Cells

In a previous study, IGSF1 purportedly attenuated activin A induction of human *FSHB* promoter-reporter activity by inhibiting ALK4 [12]. However, these studies were conducted in heterologous HEK293FT cells. In our experience, murine and porcine *Fshb* promoter-reporters are insensitive to activins in heterologous cell lines unless the transcription factors FOXL2, SMAD3, and SMAD4 are co-transfected [31]. Even under these conditions, human *FSHB* promoter-reporters are not induced by activins. Indeed, wild-type human *FSHB* reporters are poorly responsive to activins

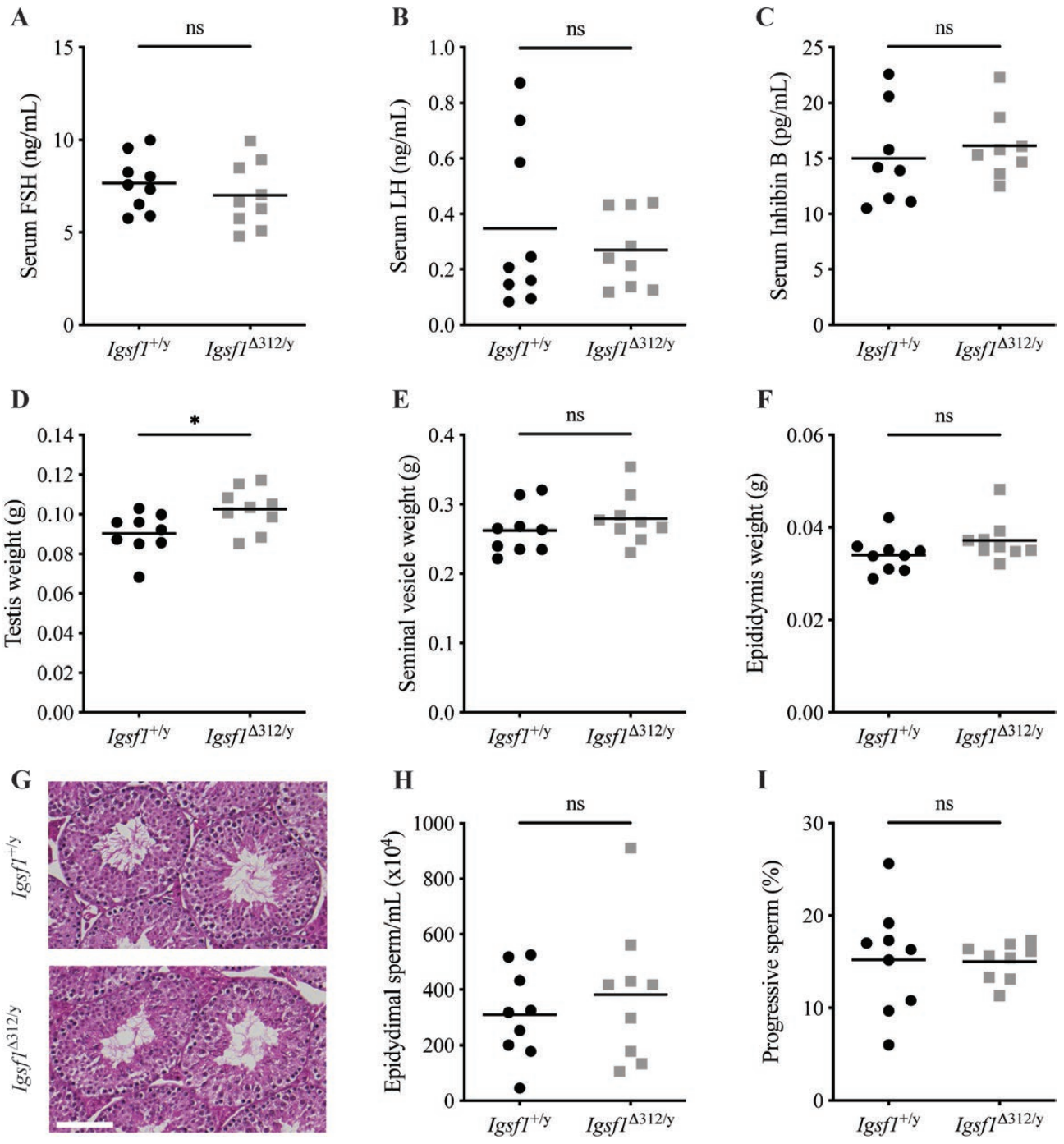

Figure 1. Normal reproductive phenotypes in *Igsf1*knockout males. Serum FSH (A), LH (B), and inhibin B (C) levels in adult male wild-type (*Igsf1*$^{+/y}$) and *Igsf1* knockout (*Igsf1*$^{\Delta 312/y}$) mice. Testis (D), seminal vesicle (E), and epididymal weights (F). Representative testicular tissue sections stained with H&E from *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ mice (G). Cauda epididymal sperm concentration (H) and percent progressive sperm from cauda epididymides (I). Samples are from 10- to 12-week-old *Igsf1*$^{+/y}$ (black) and *Igsf1*$^{\Delta 312/y}$ (gray) males. Individual data points are plotted as circles or squares; means are shown by horizontal lines. Scale bar, 100 μm. The data were analyzed by a two-tailed unpaired *t* test with Welch's correction. Abbreviations: n.s., no significant difference. * $P < 0.05$.

even in homologous LβT2 gonadotrope-like cells [31]. Therefore, we examined the effects of IGSF1 on activin A induction of a wild-type murine *Fshb*-luc reporter and a modified human *FSHB*-luc reporter in LβT2 cells. The latter reporter has a point mutation (C-165T) that increases FOXL2 binding and activin sensitivity in these cells [31]. Activin A induced transcription from both reporters, but this was unaffected by IGSF1 co-transfection (Fig. 4A-4B; ie, there was no main effect of IGSF1 in the statistical analysis).

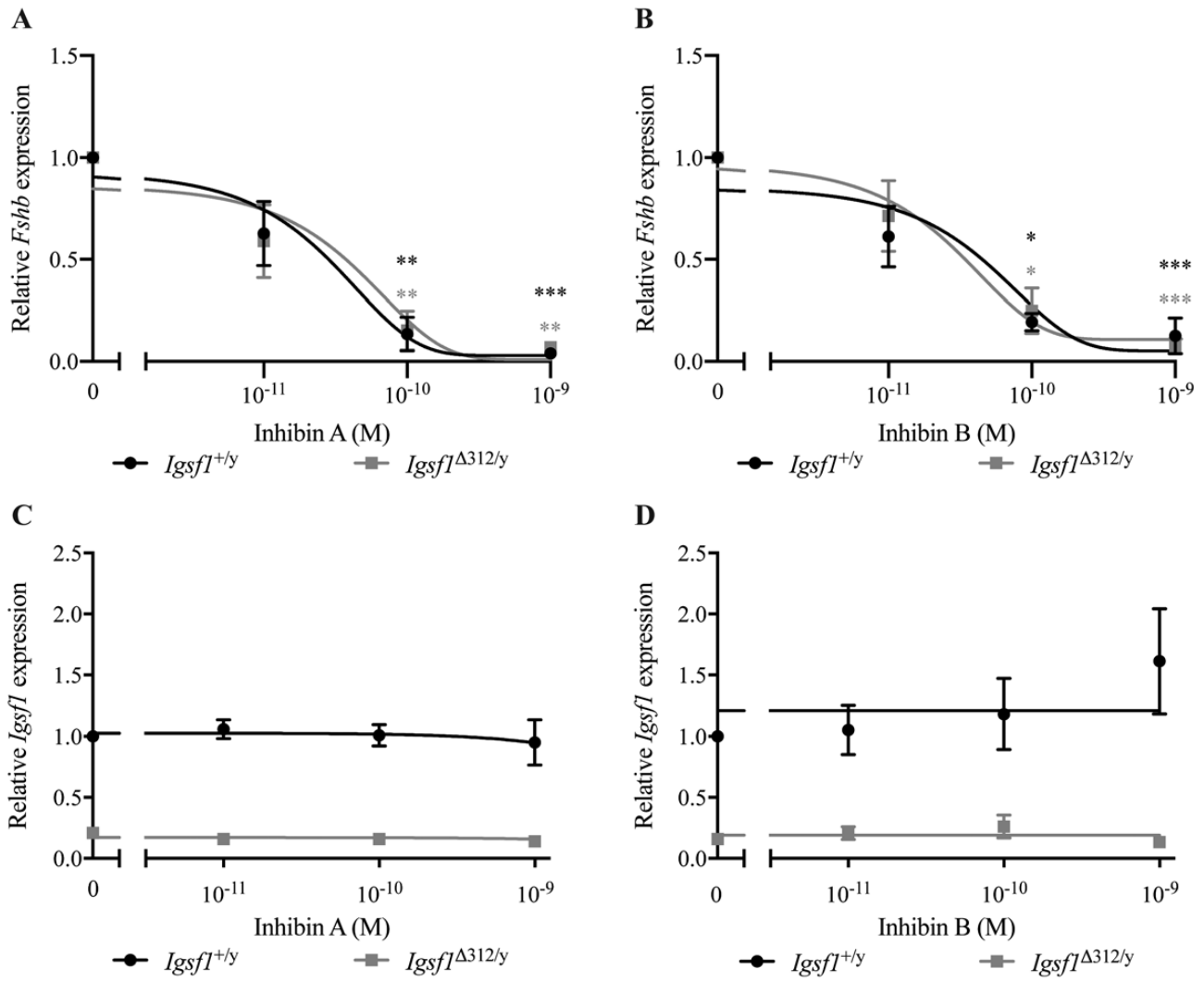

Figure 2. Inhibin A and B dose dependently inhibit *Fshb* expression in pituitaries from *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ mice. Pituitaries were isolated from 8- to 12-week-old *Igsf1*$^{+/y}$ and *Igsf1*$^{\Delta 312/y}$ males. Cells were treated with the indicated amounts of inhibin A (A and C) and inhibin B (B and D). *Fshb* (A and B) or *Igsf1* (C and D) mRNA expression was measured by RT-qPCR. *Rpl19* was used as the housekeeping gene. Data represent mean values (± SEM) from 3 independent experiments. Data were log transformed and analyzed by one-way ANOVA, followed by Holm-Sidak correction. Each treatment group was compared to its own no-treatment control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

CAGA-luc Is Not a Surrogate for *Fshb/FSHB*-luc Reporters

Another concern with the previous study [12] was that the majority of promoter-reporter experiments were performed with CAGA-luc, which the authors used as a "surrogate" for human *FSHB*-luc. Consistent with their results, we observed that activin A induction of CAGA-luc activity was inhibited by co-transfected IGSF1 in HEK293 cells (Fig. 5A). Both CAGA-luc and *Fshb*-luc (murine and porcine, but not human) reporters are SMAD3/4-responsive, but the *Fshb* reporters also require FOXL2 for their activation [31, 41, 42]. Therefore, it seemed unlikely to us that CAGA-luc could act as a true surrogate for *Fshb/FSHB* promoter-reporters. We therefore compared activation of the different reporters in response to ALK4TD with and without different combinations of SMAD3, SMAD4, and FOXL2 in heterologous HEK293 cells. ALK4TD alone strongly stimulated CAGA-luc (Fig. 5D), but not murine *Fshb*-luc (Fig. 5B) or human *FSHB*-luc with the C-165T mutation (Fig. 5C). ALK4TD induction of murine *Fshb*-luc required the co-expression of FOXL2 and SMAD3 (with or without SMAD4) (Fig. 5B). ALK4TD induction of CAGA-luc was generally unaffected by the co-expression of SMADs and FOXL2, except for the combination of FOXL2 and SMAD4, which inhibited the response (Fig. 5D). No combination of ALK4TD, SMAD3, SMAD4, and FOXL2 induced the mutant (C-165T; Fig. 5C)

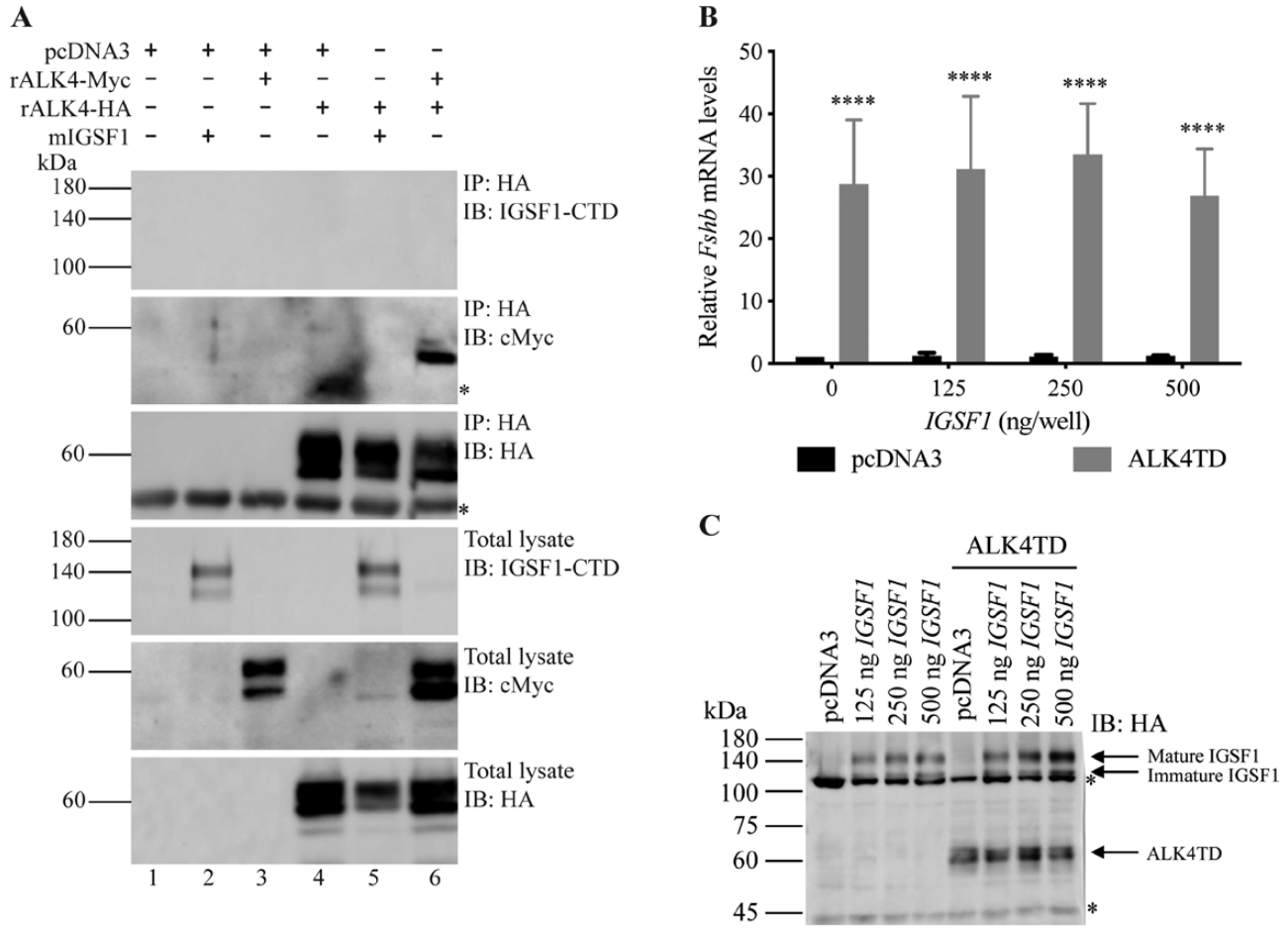

Figure 3. IGSF1 does not physically or functionally interact with ALK4. (A) HEK293 cells were transfected with a combination of pcDNA3.0, untagged IGSF1, and myc- and/or HA-tagged ALK4. Cell lysates were immunoprecipitated (IP) using an HA antibody. Immunoprecipitated and total cell lysate proteins were resolved by SDS-PAGE and immunoblotted (IB) against c-myc, HA, or IGSF1. (B and C) LβT2 cells were transfected with 0, 125, 250, or 500 ng of HA-IGSF1 with or without 500 ng HA-ALK4TD. (B) Endogenous *Fshb* expression was measured by RT-qPCR. (C) Protein lysates were resolved by SDS-PAGE and blotted with HA. In (B), bars represent mean values (+ SEM) from 3 independent experiments. *Rpl19* was used as the housekeeping gene. qPCR data were log transformed and analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. **** $P < 0.0001$ when compared to ALK4TD induction with the empty vector control (pcDNA3.0; 0 ng/mL in the figure). Asterisks on immunoblots indicate nonspecific bands.

or wild-type human *FSHB*-luc reporter in these heterologous cells (data not shown).

Igsf1 Is Not Expressed in Gonadotropes

Using a custom antibody, we previously reported that the IGSF1 protein is expressed in pituitary thyrotropes, lactotropes, and somatotropes, but not in gonadotropes, of mice and rats [3, 5]. A more recent report, using a commercial antibody, concluded that IGSF1 was exclusively expressed in thyrotropes and gonadotropes in rats [12]. To help resolve these inconsistencies, we first examined recently published pituitary single-cell RNA sequencing data sets in mice and rats [52, 53, 56]. In both species, highest levels of *Igsf1* mRNA (denoted by the extent of purple shading) were observed in thyrotropes, lactotropes, and somatotropes, but not in gonadotropes (Fig. 6A-6B). In these figures, each dot represents an individual pituitary cell. Clusters of cells show similar patterns of gene expression and can be assigned to different lineages based on the expression of specific genes, such as *Tshb* for thyrotropes (Thy), *Prl* for lactotropes (Lac), *Gh* for somatotropes (Som), and *Fshb* and *Lhb* for gonadotropes (Gt). See [52, 53] for specific information on how the cell clusters were defined. We then compared our custom IGSF1 antibody and the commercial IGSF1 antibody in western blotting with protein lysates from cells overexpressing human or murine IGSF1 or with pituitary protein lysates from

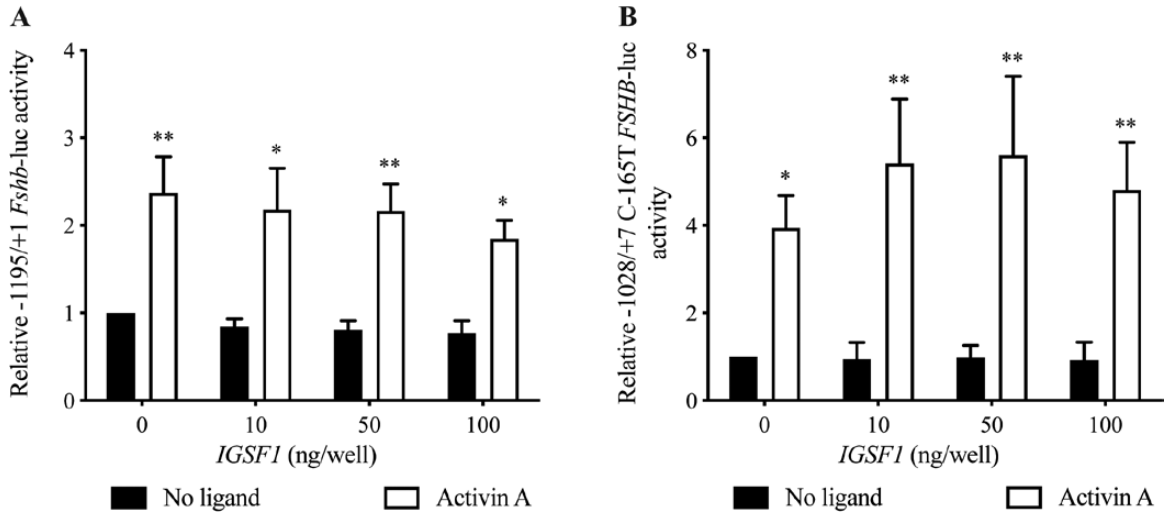

Figure 4. IGSF1 does not regulate activin A-induced *Fshb*-luc or *FSHB*-luc promoter-reporter activity. LβT2 cells were transfected with 225 ng of the murine –1195/+1 *Fshb*-luc (A) or the human –1028/+7 C-165T *FSHB*-luc (B) reporter plasmids and the indicated amount of IGSF1 expression vector. Cells were treated with either no ligand (black bars) or 25 ng/mL activin A (white bars) for 24 hours. Bars represent mean values (+ SEM) from 4 independent experiments. Data were log transformed and analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. * $P < 0.05$; ** $P < 0.01$ when comparing all conditions to no ligand/no *IGSF1* control.

adult male mice and female rats. Both antibodies could detect IGSF1 from all 3 species (Fig. 6C-6D). However, our custom antibody appeared to be more sensitive than the commercial antibody in its ability to detect IGSF1 in rodents (compare lanes 5-10 in Fig. 6C-6D, particularly in the long exposure).

Discussion

Sperm concentrations are normal in IGSF1-deficient men and male mice. This suggests that macroorchidism in IGSF1 deficiency is unlikely to result from increases in Sertoli cell proliferation, as sperm counts and Sertoli cell number are highly correlated [57]. This result is perhaps unsurprising in mice, as FSH levels are not increased in *Igsf1* knockout animals. However, there is a tendency for FSH to be elevated, particularly relative to LH, in IGSF1-deficient men. Whether or how this modest increase contributes to testicular enlargement is unclear. Hypothyroidism has also been linked to increased testis size. Nevertheless, treatment with thyroid hormone does not prevent macroorchidism in IGSF1-deficient patients. It is possible that thyroid hormone availability or actions in the testes might be compromised, but we would contend that cell autonomous roles for IGSF1 in the testis should be considered. The IGSF1 protein is expressed in both human and rat but not murine testis [3, 5]. A rat model of *Igsf1* deficiency may prove valuable in determining the causes of testicular enlargement in this disorder. Though testis size is increased in *Igsf1* knockout mice, the effect size is small, and it disappears if one corrects for the increased body mass in these animals [4].

Initial reports indicated that IGSF1 was an inhibin B co-receptor [13, 14] and, more recently, a negative regulator of activin-regulated *FSHB* transcription [12]. The data presented here challenge both of these ideas. Not only do inhibins fail to bind IGSF1 [18], but inhibin A or B suppression of FSH synthesis was unaltered in pituitaries of *Igsf1* knockout mice (Fig. 2). Although we replicated the observation that IGSF1 can inhibit activin A induction of a SMAD3/4-responsive promoter-reporter (CAGA-luc) in heterologous HEK293 cells [12] (Fig. 5A), we failed to detect a physical interaction between IGSF1 and ALK4 (Fig. 3A). More importantly, however, IGSF1 did not affect *Fshb* transcription in homologous LβT2 cells. Neither induction of endogenous *Fshb* mRNA expression by a constitutively active form of ALK4 (Fig. 3B) nor activin A stimulation of murine or human *Fshb/FSHB* promoter-reporters were affected by IGSF1 overexpression (Fig. 4). These observations call into doubt the legitimacy of using the CAGA-luc reporter as a surrogate to understand mechanisms of activin regulation of *Fshb* transcription [12]. Indeed, CAGA-luc and *Fshb/FSHB*-luc were markedly different in their regulation by SMAD proteins, FOXL2, and constitutively active ALK4 (Fig. 5B-5D).

Finally, it is unclear how IGSF1 would mediate the actions of inhibins or activins on FSH, as *Igsf1* mRNA and IGSF1 protein do not appear to be expressed in

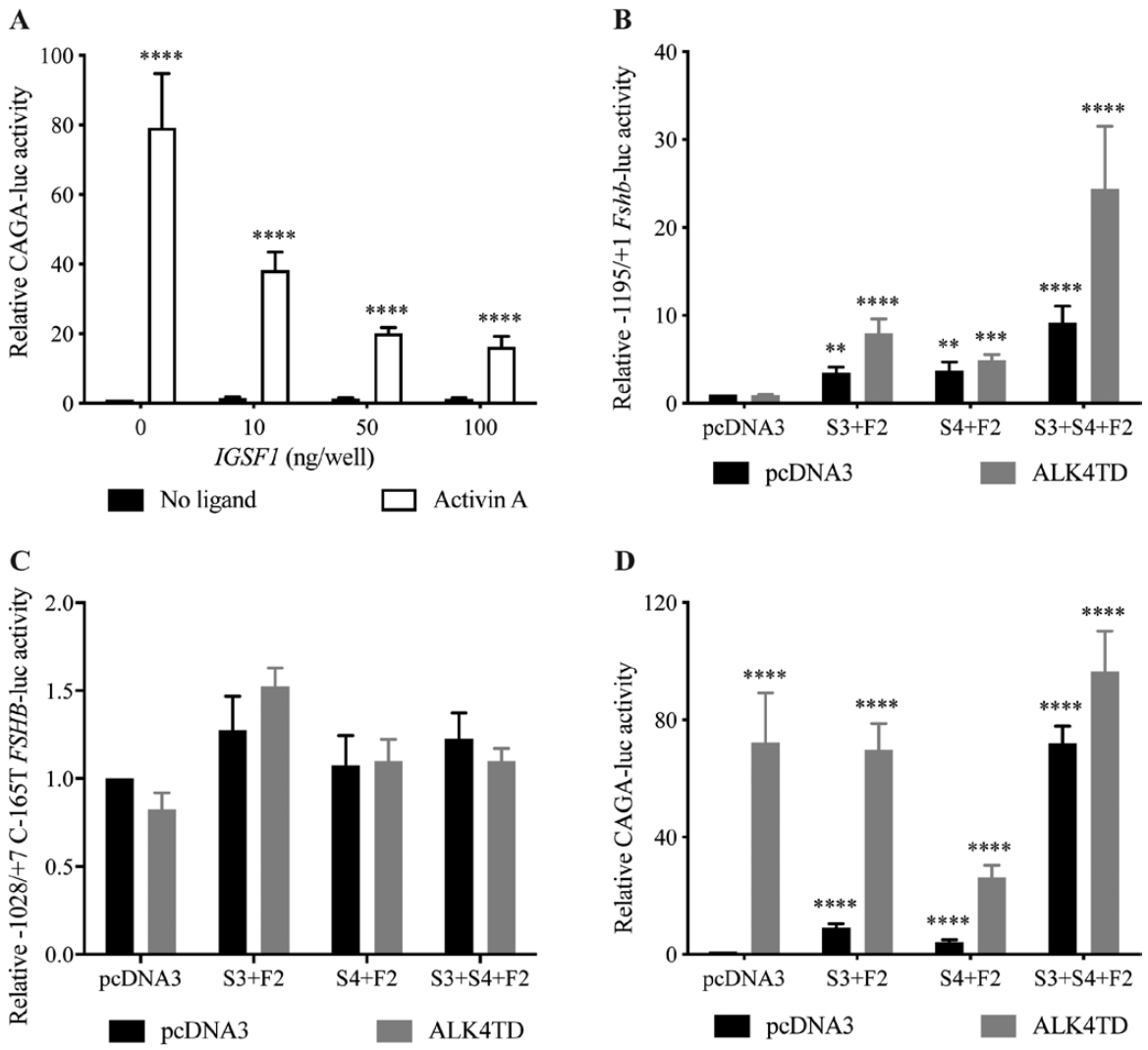

Figure 5. *Fshb*-luc, *FSHB*-luc, and CAGA-luc promoter-reporters are differently regulated by SMAD3, SMAD4, and FOXL2 in heterologous cells. HEK293 cells were transfected with the indicated amounts of IGSF1, and/or a combination of pcDNA3.0, ALK4TD, SMAD3 (S3), SMAD4 (S4) and/or FOXL2 (F2) and 225 ng of either the CAGA-luc (A and D), murine –1195/+1 *Fshb*-luc (B), or human –1028/+7 C-165T *FSHB*-luc (C). In panel (A), cells were treated with either no ligand (black bars) or 25 ng/mL activin A (white bars) for 24 hours. Bars represent mean values (+ SEM) from 4 independent experiments. Data were log transformed and analyzed using two-way ANOVA followed by Dunnett's correction. Each group was compared to pcDNA3.0 control. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

gonadotropes [3, 5, 52, 53, 56]. IGSF1 immunoreactivity was reported in rat gonadotropes [12]. We therefore tested the commercial IGSF1 antibody used in that study. Though the antibody detected IGSF1 protein in murine and rat pituitaries (by western blot; Fig. 6C), it did so less well than our custom IGSF1 antibody (Fig. 6D). The latter failed to detect the protein in gonadotropes of either species (by immunofluorescence or immunohistochemistry) [3, 5]. These data are supported by recent single-cell RNA sequencing analyses, which detected *Igsf1* mRNA in somatotropes, lactotropes, and thyrotropes, but not gonadotropes, in both species (Fig. 6A-6B) [52, 53, 56]. Collectively, our data demonstrate that IGSF1 does not regulate FSH production by altering inhibin or activin actions in gonadotropes.

In summary, the etiology of macroorchidism in IGSF1-deficient men remains unknown. From our current data, we cannot conclude whether Sertoli cell number or function is altered, but sperm production appears normal. Future investigations should focus on intratesticular functions of IGSF1 to understand if and how the loss of the protein leads to testicular enlargement.

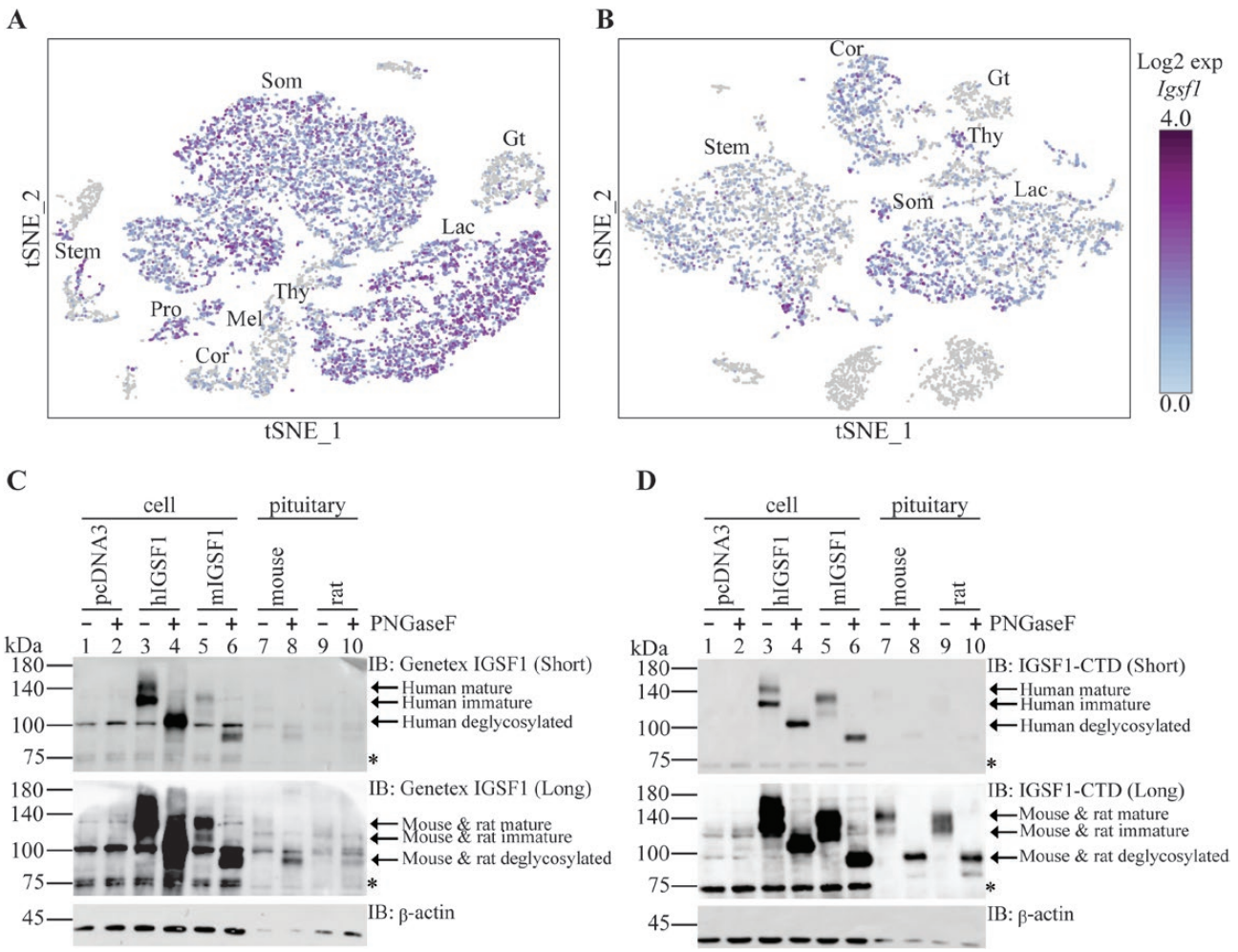

Figure 6. *Igsf1* is not expressed in rodent gonadotropes. tSNE representation of male mouse (A) and rat (B) single-cell *Igsf1* mRNA expression. Pituitary cell types are labeled above clusters: gonadotropes (Gt), somatotropes (Som), lactotropes (Lac), thyrotropes (Thy), corticotropes (Cor), melanotropes (Mel), stem cells (Stem), and proliferating cells (Pro). (C and D) Protein lysates from HEK293 cells transfected with pcDNA3.0, human (h) IGSF1, or murine (m) IGSF1 expression vectors or from murine and rat pituitaries were treated with PNGaseF and immunoblotted using commercial (C) and custom (D) IGSF1 antibodies. Blots were subjected to both short (top panels) and long (middle panels) exposures. β-actin was used as a loading control (bottom panels). Arrows mark the mature and immature glycoforms of IGSF1 and the deglycosylated bands. Asterisks on immunoblots indicate nonspecific bands.

Acknowledgments

The authors would like to thank the patients who kindly agreed to participate in this study; Dr. Bernard Robaire for providing access to the CASA system; Dr. Terry Hébert for providing HEK293 cells and rats for pituitary collection; and the late Dr. Alan Schneyer (Fairbanks Pharmaceuticals) for providing the inhibin B.

Author Contributions: C.A.H., S.D., J.M.W., and A.S.P.vT. contributed to the collection of patient data. E.B., G.S., and Y.L. contributed to mouse tissue collection and CASA. E.B. and G.S. performed the sperm counts. Y.L. conducted the LH ELISA and Y.W. conducted the FSH assay. X.Z. helped with the primary culture experiments. E.B. performed most of the in vitro experiments. C.L.S. performed the immunoprecipitation. E.B., C.A.H., D.J.B., and A.S.P.vT. designed the experiments and wrote the manuscript. All authors reviewed and approved the final version.

Financial support. This work was financially supported by operating grants to D.J.B. (Canadian Institutes of Health Research (CIHR) MOP-133557 and PJT-162343, and Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant 2015-05178) and an AUMC Foundation grant to (E.F.). E.B. and G.S. hold Doctoral Research Awards from NSERC and CIHR, respectively. C.L.S. holds a fellowship from the McGill University Faculty of Medicine.

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Disclosures: The authors have nothing to disclose. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data Availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

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