The NOTCH Ligand JAG1 Regulates GDNF Expression in Sertoli Cells

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In the seminiferous epithelium of the testis, Sertoli cells are key niche cells directing proliferation and differentiation of spermatogonial stem cells (SSCs) into spermatozoa. Sertoli cells produce glial cell line-derived neurotrophic factor (GDNF), which is essential for SSC self-renewal and progenitor expansion. While the role of GDNF in the testis stem cell niche is established, little is known about how this factor is regulated. Our previous studies on NOTCH activity in Sertoli cells demonstrated a role of this pathway in limiting stem/progenitor cell numbers, thus ultimately downregulating sperm cell output. In this study we demonstrate through a double-mutant mouse model that NOTCH signaling in Sertoli cells functions solely through the canonical pathway. Further, we demonstrate through Dual luciferase assay and chromatin immunoprecipitation quantitative polymerase chain reaction (ChIP-qPCR) analysis that the NOTCH targets HES1 and HEY1, which are transcriptional repressors, directly downregulate GDNF expression by binding to the *Gdnf* promoter, thus antagonizing the effects of FSH/cAMP. Finally, we demonstrate that testicular stem/progenitors cells are activating NOTCH signaling in Sertoli cells in vivo and in vitro through the NOTCH ligand JAG1 at their surface, indicating that these cells may ensure their own homeostasis through negative feedback regulation.

Keywords: GDNF, JAG1, NOTCH, Sertoli cell, spermatogenesis, spermatogonia

Introduction

IN THE RODENT TESTIS, mature sperm cells originate from self-renewing spermatogonial stem cells (SSCs) called A_{single} spermatogonia, which reside in the basal part of the seminiferous epithelium, in contact with the basement membrane. Stem cells differentiate into progenitors called A_{paired} and A_{aligned} spermatogonia that remain connected by intercellular bridges. A_{aligned} spermatogonia proliferate and differentiate into more mature germ cells such as A_1 – A_4 spermatogonia, type B spermatogonia, and spermatocytes that will undergo meiosis and spermiogenesis (for a review, see Jan et al. [1]). A_{single} , A_{paired} , and A_{aligned} are sometimes collectively referred as undifferentiated spermatogonia [2]. Within the niche, self-renewal and differentiation of SSCs depend on their close association with somatic cells such as Sertoli cells, peritubular cells, and interstitial cells, which produce specific growth factors including glial cell line-derived neurotrophic factor (GDNF) [3–6].

GDNF belongs to a family of growth factors that also includes neurturin (NTN), persephin (PSPN), and artemin (ARTN). These four proteins are closely related to transforming growth factor (TGF- β , TGF β) and exert their biological activities by activating the RET transmembrane

receptor tyrosine kinase via the GFRA co-receptor [7,8]. GDNF/RET signaling regulates processes such as cell survival, proliferation, differentiation, migration, chemotaxis, and branching morphogenesis [9]. As a pleiotropic factor, GDNF is therefore essential to multiple developmental processes including the formation of the enteric nervous system, synaptic plasticity, migration of neural crest cells, kidney formation, and maintenance of fetal germ cells [10–13]. Consequently, global GDNF and RET knockout mice die shortly after birth. In addition to its role in fetal development, GDNF in the adult is crucial for the survival of dopaminergic and motor neurons [14,15], and it is currently used in clinical trials to treat advanced Parkinson disease. In the brain, GDNF is mainly produced by neurons themselves, and it appears to act through a positive feedback loop [16]. In the testis, GDNF is a recognized trophic factor that is critical for SSC self-renewal, and its requirement for maintaining an adequate pool of SSCs in the testis throughout the life of a male is indisputable [3,17–19]. In the testis, GDNF is produced by two niche cells, Sertoli cells and peritubular myoid cells [6,20–22].

While the role of GDNF in the neuronal and SSC niches is established, we know little about how this factor is

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regulated. In the brain, GDNF production by glial cells is enhanced by agonists of metabotropic glutamate receptors [23,24]. In the mouse testis, GDNF expression by Sertoli and peritubular cells might depend on levels of gonadotropic pituitary hormones (LH and FSH) and testosterone, but reports are conflicting and additional data will be necessary to fully understand the mechanisms of GDNF production [22,25–27]. Because sperm is produced throughout the life of males, a steady demand of self-renewing stem cells needs to be met by an appropriate amount of GDNF and other growth factors that are dispensed by niche cells. However, previous work not only demonstrated that lack of GDNF in the testis led to sterility, but also that excess of GDNF caused germ cell hyperplasia and ultimately testicular tumors in mice [3,17,28]. Therefore, understanding how GDNF is regulated, positively or negatively, is essential to understand germ cell homeostasis and fertility problems.

Using gain- and loss-of function models, we recently demonstrated that NOTCH signaling in Sertoli cells might be crucial for germ cell homeostasis. An excess of NOTCH activity in the fetal gonad led to loss of prenatal germ cells, while lack of NOTCH activity increased the number of postnatal germ cells [29,30]. These effects are possibly caused by changes in *Gdnf* expression levels, which appear inversely correlated to NOTCH activation at the messenger RNA (mRNA) level. However, a conclusive link between NOTCH signaling activity and inhibition of *Gdnf* expression was still lacking.

NOTCH signaling is a highly conserved juxtacrine signaling pathway involved in a variety of cell fate decisions key to the development and maintenance of multiple organ and tissue systems. NOTCH receptors (NOTCH1–4) are activated by contact with membrane-bound ligands on neighboring cells, such as JAGGED (JAG) and DELTA (DLL). Upon activation of the canonical pathway, the NOTCH intracellular domain (NICD) is cleaved and translocates to the nucleus where it associates with a DNA-binding protein called “recombining binding protein suppressor of hairless” (RBPJ), also called CSL [31]. This displaces a repressor complex from the RBPJ protein. Components of an activation complex, such as MAML1 and histone acetyltransferases (HAT), are then recruited to NICD-RBPJ, triggering the conversion of RBPJ from a transcriptional repressor to a transcriptional activator [31]. In the canonical pathway, targets of RBPJ include the HES/HEY family of transcriptional repressors [32,33]. Mammalian HES/HEY proteins fulfill important roles during development and adulthood, but the result of their activation (proliferation, differentiation, or lateral inhibition) depends on the cell type [34,35].

In this study, we demonstrate that the production of GDNF by Sertoli cells is under the regulatory control of the canonical NOTCH signaling pathway, and we provide evidence that the NOTCH target proteins HES1 and HEY1 directly repress GDNF expression. Further, we demonstrate that the NOTCH ligand JAG1 is the main activator of NOTCH signaling and is highly expressed by undifferentiated spermatogonia in comparison to other germ cells, establishing that these cells participate in the activation of NOTCH signaling and GDNF modulation within the stem cell niche.

Materials and Methods

Mice and breeding schemes

Mice were housed in accordance with NIH guidelines and experimental protocols were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Texas MD Anderson Cancer Center and Texas A&M University, Institute of Biosciences and Technology.

Mvh^{Cre-mOrange/+} mice, which express the mOrange fluorescent protein in all their germ cells, were kindly given to us by Dr. David Page, Whitehead Institute for Biomedical Research (Cambridge, MA) [36]. To specifically target/express genes following Cre-mediated recombination in Sertoli cells, we used *Tg(AMH-cre)1Flor* mice (or *AMH-cre* mice) provided by Dr. Paul Cooke (University of Florida, Gainesville, FL) [37,38]. To express TdTomato following Cre-mediated recombination in germ cells and Sertoli cells, we used *Gt(ROSA)26Sor^{tm14(CAT-tdTomato)Hze}* mice obtained from the Jackson Laboratory (Bar Harbor, ME) [39].

Because it is well known that NICD can have noncanonical, RBPJ-independent functions [40], we generated NOTCH gain-of-function mice (*Amh-cre;Rosa^{NICD/+}*) on a background of Sertoli cell-specific RBPJ ablation (*Amh-cre;Rbpj^{fl/fl}*). We therefore crossed homozygous *Rosa26^{NICD/NICD}* mice (containing alleles for NOTCH overexpression) with female *Rbpj* knockout mice that also harbor both alleles for Cre-mediated YFP⁺ expression (*Amh-cre;Rbpj^{fl/fl};Rosa^{YFP/YFP}*). RBPJ is a key element in the NOTCH pathway. After two generations of breeding, the rescue breeding colony was established with male and female breeders of the following genotypes, respectively: *Rbpj^{fl/fl};Rosa^{NICD/NICD}* (male) and *Amh-cre;Rbpj^{fl/fl};Rosa^{YFP/YFP}* (female). Resulting offspring from these breeding include controls (*Rbpj^{fl/fl};Rosa^{NICD/YFP}*) and Rescues (*Amh-cre;Rbpj^{fl/fl};Rosa^{NICD/YFP}*).

To obtain an enhanced marker facilitating germ cell fluorescence-activated cell sorting (FACS) isolation, *Mvh^{Cre-mOrange/+}* mice were crossed with *Rosa26^{flSTOP-tdRFP}* to obtain *Mvh^{Cre-mOrange/+};Rosa26^{flSTOP-tdRFP}* mice, or *Mvh-RFP* mice. To facilitate Sertoli cell FACS isolations, we used *Amh-cre;Rbpj^{+/+};Rosa26^{YFP/YFP}* mice, which were obtained as previously described [30]. For spermatocyte isolations, we FACS-sorted germ cells highly expressing GFP from *Tg(Dazl-EGFP)10Rarp* obtained from the Jackson Laboratory [41].

Histological analysis

Periodic acid/Schiff-stained testis cross sections were examined for stages of spermatogenesis by applying standard staging criteria [42]. A total of 150 tubules per animal from a minimum of two separate testis sections per animal were staged and the number of large, luminal residual bodies present in stage IX and X tubules was recorded. A mean value of the number of residual bodies found within staged tubules was assigned to an individual mouse before the final calculation on a per mouse basis was carried out.

Further, the numbers of round spermatids and pachytene spermatocytes per Stage VII round tubule cross section at postnatal day 70 (P70) were manually counted within the epithelium of a minimum of 20 tubules per animal from a minimum of two separate testis sections per animal. Mean values on a per mouse basis were used for final calculations.

Germ cell isolations

Mvh-RFP mice were used at 6–10 dpp to isolate germ cells by a two-step enzymatic digestion [43] followed by FACS. GFRA1-positive cells were further obtained using 2 µg/mL of a rabbit anti-GFRA1 primary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) coupled with a FITC-conjugated anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA; RFP⁺-FITC⁺ cells). KIT-positive germ cells (differentiating spermatogonia) were sorted with 0.8 µg/mL of a rat anti-KIT antibody conjugated with APC (BD Biosciences, Franklin Lakes, NJ; RFP⁺-APC⁺ cells) (all antibodies are listed in Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). *Tg(Dazl-EGFP)10Rarp* adult mice were used to isolate DAZL-positive germ cells by FACS, which were further fractionated into KIT-positive differentiating spermatogonia (EGFP⁺-APC⁺ cells) and preleptotene spermatocytes, and KIT-low/negative leptotene spermatocytes (EGFP⁺-APC⁻ cells). Germ cells were FACS-sorted on a BD Influx cell sorter (BD Biosciences). Isolated germ cells were used to quantify expression of NOTCH ligands by quantitative polymerase chain reaction (qPCR).

Busulfan treatment

To assess the importance of germ cells in activating NOTCH signaling, we treated 8-week-old *Amh-cre;Rbpj^{+/+}; Rosa26^{YFP/YFP}* mice with 40 mg/kg busulfan (Sigma, St Louis, MO) [44]. These young adult mice were used to ensure that Sertoli cells, which are not dividing after puberty, are not affected by busulfan. Two months later, testes were collected from treated ($n=3$) and untreated ($n=3$) mice and Sertoli cells isolated according to a standard two-step isolation procedure [43]. Sertoli cells were FACS-sorted using a 550/30-nm band pass filter for YFP on a BD Influx cell sorter (BD Biosciences), and their expression of NOTCH target genes and *Gdnf* quantified by qPCR. Experiments were repeated three times.

Sertoli cell cultures

Matrigel-coated dishes and plates were prepared according to previously published procedures [29,30,45]. Briefly a 1:5 dilution of Matrigel (BD Biosciences) in Dulbecco's modified Eagle's medium (DMEM)/F12 was added to culture dishes or wells and incubated for 30 min at 4°C. Excess media was removed and the dishes were incubated at 37°C in a humidified incubator to allow Matrigel to solidify. Sertoli cells from *Amh-cre;Rbpj^{+/+};Rosa26^{YFP/YFP}* mice were isolated at 3 and 6 dpp following a standard two-step digestion procedure [43]. Because adult Sertoli cells do not proliferate, prepubertal Sertoli cells were chosen for their ability to be maintained and expanded in Matrigel cultures for over a week [45,46]. Cells were plated at a concentration of 3×10^5 cells in Matrigel-coated 24-well plates and grown for 4 days in D-MEM/F12 medium. Alternatively, cells were plated at a concentration of 3×10^6 cells in 100 mm diameter Matrigel-coated plates. Cells were then trypsinized and reseeded for the appropriate experimental conditions as described below.

In vitro ligand activation of NOTCH signaling

For ligand activation experiments, Pierce[™] Protein A/G Coated Plates (ThermoFisher Scientific, Grand Island, NY)

were treated either with phosphate-buffered saline (PBS, controls) or recombinant rat JAG1-Fc Chimera Protein (R&D Systems, Minneapolis, MN) diluted in PBS to desired concentrations(s) and incubated overnight at 4°C. Under these conditions, the ligand adheres to the surface of the plate in the proper position, allowing optimal activation of the NOTCH receptors. The next day, PBS or PBS+ligand was removed from the wells, and a 1:5 dilution of Matrigel (BD Biosciences) in DMEM/F12 was added on top of the immobilized ligand for 30 min at 4°C. Excess Matrigel was removed and the plates were incubated at 37°C for another 30 min to allow Matrigel to solidify. Sertoli cells were seeded (50,000/well) into each ligand- or control-containing well, cultured in DMEM/F12 in a humidified 5% CO₂ incubator and subjected to mRNA isolation and qPCR analysis 48 h later.

RNA interference

Sertoli cells from wild-type (WT) testes were seeded at a concentration of 5×10^4 cells into 96-well plates coated with 10 µg/mL JAG1-Fc Chimera protein and Matrigel as described above. The cells were then transfected with *Hes1* or *Hey1* small interfering RNAs (siRNAs), mock, or scrambled siRNAs (Invitrogen) using RNAiFect kit according to manufacturer's instructions (Qiagen, Valencia, CA). RNA was isolated after 24 or 48 h to measure the levels of gene expression by qPCR.

Quantitative gene expression analysis

For Sertoli cells cultured in microtiter plates, RNA extraction, reverse transcription, and qPCR were performed using the TaqMan Gene Expression Cells-to-CT Kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. For Sertoli cells grown in 24-well plates or culture dishes, RNA was extracted using a buffer containing 1 M Tris-HCl (Sigma), 10% IGEPAL CA-630 (Sigma), and 5 M NaCl in RNase-free H₂O [47]. One step reverse transcriptase (RT)-qPCR was performed with the qScript XLT One-Step RT-qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD). Relative quantitative fold change was determined using the $\Delta\Delta C_t$ method. In all analyses, the expression value of each gene was normalized to the amount of an internal control gene (*Eif3l* or *Rps3*) complementary DNA (cDNA) to calculate a relative amount of RNA in each sample [29,48]. qPCR was carried out with at least three biological replicates per group, and each biological replicate was carried out in duplicate or triplicate per plate. The raw critical threshold values of technical replicates were averaged before mean, standard error, and statistical analysis was determined for the biological replicates. The TaqMan assays used for specific transcripts are listed in Supplementary Table S2.

Dual luciferase reporter assay

Two different nucleotide sequences containing E- and N-boxes upstream of the transcription start site 1 (TSS1) of the mouse *Gdnf* proximal promoter [49] were cloned into pGL3-Basic (Promega, Madison, WI). Briefly, sequence restriction analysis was carried out using Webcutter to identify appropriate restriction sites. Two primer pairs were designed with addition of a Kpn1 restriction site toward the 5' end and a Xho 1 restriction site toward the 3' end of each

sequence. Promoter sequences were amplified using PCR with mouse's tail DNA as template and cloned into the same restriction sites of the firefly luciferase pGL3-Basic reporter vector using T4 DNA ligase (Promega). Plasmids were amplified by transformation of Stellar[™] competent cells (Clontech, Mountain View, CA). Several bacterial colonies were selected with ampicillin, and DNA sequenced to ensure proper construct insertion. Promoter sequence 1 (GDNF-1.0 kb, bp -1,040 to +132) contains three E-box motives allowing HEY1 binding at bp -771 to -326. Promoter sequence 2 (GDNF-1.5 kb, bp -1,539 to +132) contains in addition two N-boxes and one E-box in addition to two CT-rich regions (CTRR) between bp -1,500 and -1,369 allowing specific binding of HES1 and HEY1 (Fig. 2A).

Because transfection efficiency in primary cells is notoriously low, which we confirmed for Sertoli cells, we used human embryonic kidney HEK-293 cells for these experiments. HEK-293 cells were seeded in 96-well plates at a concentration of 10,000 cells/well. They were cultured in DMEM containing 10% fetal bovine serum (Gibco/Invitrogen), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco/Invitrogen). The cells were incubated until 80% confluence in a humidified incubator at 37°C and 5% CO₂. Ten micrograms per milliliters of each pGL3-Basic reporter construct was transfected into the cells using FuGENE HD transfection reagent (Promega), together with 1 µg/mL of Renilla luciferase reporter vector pRL-TK (Promega) as internal control. Each treatment was performed in triplicate for a total of three experiments. Cells were collected 48 h after transfection and lysed. Luciferase activities were directly measured using the Dual Luciferase Reporter Assay System (Promega) and a LumiStar Galaxy luminometer (BMG-Labtech, Cary, NC) according to the Promega kit instructions. Firefly luciferase activity was normalized to Renilla luciferase activity. The empty pGL3-Basic vector served as a blank control. Triplicate samples were used for each point in three independent experiments.

Chromatin immunoprecipitation-qPCR

Approximately 1×10^6 HEK-293 cells or mouse primary Sertoli cells were grown to 95% confluence in DMEM/F12 serum-free culture media supplemented with 100 U/mL penicillin and 100 U/mL streptomycin. The cells were washed twice with PBS and protein-DNA cross-linked with 1% formaldehyde for 15 min at room temperature. Formaldehyde was quenched by the addition of 0.125 M glycine (Sigma) for 5 min. Cells were then washed twice with ice-cold PBS containing 0.1 M PMSF (Sigma) and 1× of a standard protease inhibitor cocktail (Halt; Thermofisher Scientific). Cells were scraped and resuspended in 1 mL PBS containing 0.25% Triton X, 10 mM EDTA, and 10 mM HEPES, with a final pH of 7.9. Samples were incubated for 15 min and centrifuged at 4°C and 1,000g to obtain pellets. Pellets were resuspended in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 1× Halt protease inhibitor cocktail in 50 mM Tris-HCl, pH 8.1, and sonicated on ice 10 times for 30 s each, with a 30-s interval at the maximum setting (Bioruptor; Diagenode, Denville, NJ). Sonicated chromatin was diluted in lysis buffer without SDS to bring the final concentration of SDS to 0.05%. A chromatin aliquot (10 µL) was run on a 1% agarose gel to

confirm presence of desired fragments (300–600 bp). Five micrograms chromatin was incubated overnight with IP grade antibodies for HES1 (rabbit monoclonal antibody No. 11988; Cell Signaling Technology, Danvers, MA) or HEY1 (rabbit polyclonal antibody No. 19929-1-AP; Protein Tech, Rosemont, IL; Supplemental Table S1) at 4°C with rotation. As negative controls, chromatin was incubated with polyclonal rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and protein G-coated magnetic beads alone. Immunoprecipitation was performed by adding protein G-coated magnetic beads (Magnetic Dynabeads-Protein G; Invitrogen) for 1 h, then immune-complexes were precipitated with a magnetic stand. The beads were washed sequentially for 10 min each in a low salt buffer containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl in 20 mM Tris-HCl, pH 8.1, followed by a high salt buffer with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 500 mM NaCl in 20 mM Tris-HCl, pH 8.1, and finally a LiCl wash buffer containing 0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA in 10 mM Tris-HCl, pH 8.1. The beads were then washed thrice with TE buffer and extracted three times with 1% SDS, 0.1 M NaHCO₃, 0.1 mg/mL proteinase K, and 0.6 M NaCl. Eluates were reverse-cross-linked by heating at 65°C for 6 h and at 95°C for 10 min. The beads were removed and the released DNA fragments were purified with QIAquick DNA purification Kit (Qiagen). qPCR was performed with 1 µL final extract for 45 cycles of amplification with TaKaRa *Taq* polymerase (TaKaRa Bio USA, Mountain View, CA), and human/mouse primers flanking HES/HEY binding sites in the *Gdnf* proximal promoter (Supplementary Table S3). As a negative control, primers were chosen for amplification of a DNA sequence without HES/HEY binding sites 500 bp away from the binding site-rich sequences. The PCR products were visualized on 1% agarose gel (Supplementary Fig. S1). Triplicate qPCR reactions were performed with 500 nM of primers, 10 µL of 2×SYBR green I PCR master mix (Applied Biosystems), and 2 µL DNA in a total of 20 µL reaction volume. Results were computed as percent antibody bound per input DNA, then normalized to IgG controls.

Statistical analysis

All data were processed using MS Excel and GraphPad Prism 6 (GraphPad Software). Measurement data were expressed as mean ± standard error of the mean. The means of two samples were compared by using a two-tailed Student's *t*-test. Means of multiple samples were compared by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. $P < 0.05$ was considered significantly different. For chromatin immunoprecipitation (ChIP)-qPCR, additional two-way ANOVA was used for multiple comparisons between independent experiments.

Results

GDNF production in Sertoli cells is regulated by the canonical NOTCH signaling pathway

As reported previously, a NOTCH GFP-reporter mouse model (TNR-GFP) demonstrated that NOTCH signaling was activated only in Sertoli cells [29]. To understand the role of NOTCH signaling in Sertoli cells we overactivated

the pathway by constitutively overexpressing the intracellular domain of NOTCH1, NICD1 [29]. This induced sterility, caused by a complete lack of germ cells by P2 while Sertoli cells remained functionally normal [29]. NOTCH pathway activation was accompanied by a sharp increase in levels of the NOTCH target genes *Hes1*, *Hey1*, and *HeyL*, which are transcriptional repressors. Conversely, abolishing NOTCH signaling in Sertoli cells by conditionally ablating RBPJ function in these cells led to a sharp decrease of the transcriptional repressors *Hes1*, *Hey1*, and *HeyL*, and the simultaneous increase in germ cell numbers [30]. Thus NOTCH signaling appeared to exert a negative effect on germ cell maintenance. However, it is well known that NICD can have noncanonical, RBPJ-independent functions [40]. To test this, we generated Sertoli cell specific mice constitutively expressing NICD on a background of RBPJ ablation (*Amhcre;Rbpj^{fl/fl};Rosa^{NICD}/YFP*, named “Rescues”). Data from the rescue mice demonstrate that—unlike NOTCH overactivation that induces complete lack of germ cells by P2—NOTCH overactivation in the absence of RBPJ restores fertility, although with the same functional consequences of RBPJ deletion. Rescue mice therefore revealed a phenotype similar to that of *Rbpj* loss-of-function mice. While overall body weight remained identical to WT littermates (Fig. 1A), rescue mice exhibited testes with increased testicular weight (Fig. 1B) and sizes (Fig. 1C–E) along with a percentage of adult mice (~20%) displaying late-onset testicular atrophy with tubule lithiasis (Fig. 1C, F). Tubules diameters were increased (Fig. 1G) due to a widespread increase in seminiferous epithelium thickness (Fig. 1I) caused by an increase in all germ cell numbers like in the *Rbpj* KO testes, while lumen diameters remained constant (Fig. 1H) [30]. Counting of the germ cells in stages VII tubules indicated a 100% increase in the number of pachytene spermatocytes and a 50% increase in the number of round spermatids (Fig. 1K, L). Interestingly, similar to the *Rbpj* KO testes and consistent with an increase in the number of germ cells, the number of residual bodies at stages IX and X of the seminiferous epithelium were significantly increased (Fig. 1E' red arrowheads, and Fig. 1J). Further, Sertoli cells isolated from the adult rescue mice showed a sharp decrease in expression of the NOTCH target genes *Hes1*, *Hey1*, and *HeyL* with concomitant upregulation of *Gdnf* that was near identical to the expression of these genes in Sertoli cells isolated from the adult *Rbpj* KO mice, which were isolated side-by-side (Supplementary Fig. S2). This overall phenotype indicates that, at least in Sertoli cells, the majority of the function of the activated form of NOTCH (NICD) is through binding with RBPJ and downstream activation of HES/HEY transcriptional repressors, which is indicative of the canonical NOTCH signaling pathway.

Both HES1 and HEY1 are directly binding to the GDNF promoter to downregulate its expression

Our previous data indicated that expression of the niche factor *Gdnf* varies inversely with activation of NOTCH signaling pathway in Sertoli cells, at least at the mRNA level [29,30]. Therefore, we sought to test whether there is physical interaction between HES1 and HEY1 transcriptional repressors, which are direct targets of the NOTCH pathway, and the

human and mouse *GDNF* promoters. We performed ChIP followed by qPCR analysis. The human *GDNF* promoter contains only three type B E-boxes in a region spanning bp –1,335 to –1,095 (Fig. 2A). These sequences are preferentially bound by HEY1, but they also can be used by HES1 [50]. Our data obtained with HEK293 cells are presented in Fig. 2C and show that both HES1 and HEY1 interact with the human *GDNF* promoter within –1.5 kb of the TSS using specific antibodies and two primer pairs (PP1 and PP2) flanking this region. Interaction is strong and significant compared with IgG alone (~24-fold), and the HES1 antibody retrieves ~12% of DNA input. HEY1 antibody also retrieves significant amount of DNA in comparison to IgG. We next performed ChIP-PCR using primary mouse Sertoli cells. The mouse *Gdnf* promoter contains a region between bp –1,700 and –1,350 from the transcription start TSS1 (previously defined by Lamberti and Vicini [49]) that contains several E- and N-boxes sequences (Fig. 2A). In particular, region –1,538 to –1,350, recognized by primer pairs 2 (PP2) and primer pair 3 (PP3), contains one E-box and two N-boxes (Fig. 2B) allowing strong interaction with HEY1 (Fig. 2D). We also tested the interaction of HES1 and HEY1 with a region containing only one E-box located at bp –439 to –320, with primer pair 1 (PP1). This region allowed weak binding of HES1 but effective binding of HEY1, consistent with HEY1 affinity for E-boxes (Fig. 2D). Additional experiments confirmed interactions of HES1 and HEY1 within the –1,538 to –1,350 bp region of the *GDNF* promoter (Supplementary Fig. S3). Therefore, our data indicate that in primary Sertoli cells, *GDNF* expression might be effectively downregulated by NOTCH signaling through activation of the transcriptional repressors HES1 and HEY1. To confirm that HES1 and/or HEY1 are downregulating *GDNF* expression, we used a standard dual luciferase reporter assay. Because transfection efficiency in primary Sertoli cells was very low (2%–3%), we only used HEK-293 cells for these experiments. HEK 298 cells were transfected with two different mouse *Gdnf* promoter regions driving luciferase. The first promoter sequence (*Gdnf*-1.0, bp –1 to –1,024 from the previously defined TSS1 [49]) contained three E-boxes that allow HEY1 binding (Fig. 2A). The second promoter sequence (*Gdnf*-1.5, bp –1 to –1,544 from TSS1) contained the E-Box/N-Box rich region (Fig. 2A, B). As shown in Fig. 3A and B, activity of the luciferase enzyme was markedly decreased only after co-transfection with a *Hes1* or a *Hey1* pCMV expression vector. For both promoter sequences, HEY1 expression strongly downregulated luciferase activity compared to HES1 expression. Together, the data presented in Figs. 2 and 3 demonstrate that *GDNF* downregulation depends on activation of the NOTCH pathway through its canonical repressors HES1 and HEY1.

NOTCH activation is inversely proportional to stage-specific GDNF expression by Sertoli cells in the seminiferous epithelium

In the mammalian seminiferous epithelium, germ cells are arranged according to well-defined cell associations called stages [42,51], and several investigators recently demonstrated stage-specific expression of *GDNF* by Sertoli cells [20,52,53]. In the mouse and rat, this expression is highest at stages IX–III and lowest at stages V–VIII. To determine whether there is

FIG. 1. Characterization of rescue mice with nonatrophic testes. Rescue mice Sertoli cells do not express a functional *Rbpj*, while they overexpress NICD1. **(A)** Body weights are not significantly different between control (*Rbpj*^{fl/fl}; *Rosa*^{NICD/YFP}) and rescue (*Amh-cre*; *Rbpj*^{fl/fl}; *Rosa*^{NICD/YFP}) mice throughout adulthood. **(B)** Nonatrophic testis weights are significantly increased in rescue mice beginning at 2 months of age, which persists throughout adulthood. **(C)** Representative images of control and nonatrophic and atrophic rescue testes at postnatal day 70 (P70). Testes with NICD1-overexpressing Sertoli cells are devoid of germ cells and have been described elsewhere [29]. Representative periodic acid–Schiff staining of control **(D)** and nonatrophic **(E)** and atrophic **(F)** rescue testes at P70. **(D'–F')** Higher magnification representative images of **(D–F)**. Quantification of tubule diameter **(G)**, lumen diameter **(H)**, and epithelial thickness **(I)** in control and nonatrophic rescue mouse testes at P70. **(J)** Quantification of the number of LLRBs (**E'**, red arrowheads) per tubule cross section at stages IX and X of the seminiferous epithelium. **(K, L)** Quantification of the number of round spermatids **(K)** and pachytene spermatocytes **(L)** at stage VII of the seminiferous epithelium. Results are given as mean ± standard error of the mean. ****P* < 0.005. Scale bar = 2 mm **(C)** and 100 μm **(D–F)**. LLRBs, large, luminal residual bodies; NICD, NOTCH intracellular domain.

truly an inverse relationship between NOTCH activity and *Gdnf* expression in the seminiferous epithelium, we used the TNR-GFP mouse model [29,54] to FACS Sertoli cells with high and low NOTCH activity (Fig. 4A), then performed gene expression analysis of different Sertoli cell markers on these fractions. Our results demonstrate that *Gdnf* expression in Sertoli cells is inversely related to NOTCH activity, itself

shown by increased expression of GFP and *Hey1* (Fig. 4B). In addition, the expression of *Rhox5* (*Pem*), a gene highly expressed during stages V–VIII of the seminiferous epithelium [55–57], is also increased when *Hey1* is increased. These data therefore demonstrate that downregulation of *Gdnf* expression at stages V–VIII of the seminiferous epithelium [20] correlates with NOTCH signaling activity.

FIG. 2. Analysis of the human and mouse *GDNF* promoter with respect to HES and HEY putative binding sites. **(A)** The human *GDNF* promoter contains three E-box sequences within a $-1,335$ to $-1,095$ bp region from the transcription start site, allowing putative binding of HEY1. The mouse *Gdnf* promoter contains a N-box/E-box-rich region for binding of HES1 and HEY1 at bp $-1,700$ to $-1,350$ and additional E-boxes downstream. The transcription start site is TSS1 [49]. **(B)** N-Box/E-box-rich region of the mouse *Gdnf* promoter with primer pairs (PP2 and PP3) used for ChIP-qPCR. **(C)** ChIP-qPCR analysis of the human *GDNF* promoter showing binding of HES1 and HEY1 in the E-box-rich region (bp $-1,335$ to $-1,095$) probed with two different primer pairs (PP1 and PP2, Supplementary Table S3). Results are given as mean $\pm$ standard error of the mean. $*P < 0.05$ and $**P < 0.01$. **(D)** ChIP-qPCR analysis of the mouse *Gdnf* promoter showing binding of HES1 and HEY1. Primer pair 1 (PP1, Supplementary Table S3) probes a region containing only one E-box (-400 to -326 bp). Primer pairs 2 and 3 (PP2 and PP3, Supplementary Table S3) probe part of the N-box/E-box-rich region (bp $-1,480$ to $-1,335$). Results are given as mean $\pm$ standard error of the mean. $*P < 0.05$ and $**P < 0.01$. ChIP-qPCR, chromatin immunoprecipitation quantitative polymerase chain reaction; GDNF, glial cell line-derived neurotrophic factor; TSS1, transcription start site 1.

FIG. 3. HES1 and HEY1 transcription factors down-regulate GDNF expression in a Dual luciferase reporter assay. Two different nucleotide sequences containing E- and N-boxes upstream of the TSS1 of the mouse *Gdnf* proximal promoter were cloned into pGL3-Basic vector and co-transfected with *Hes1*- or *Hey1*-pCMV expression vectors into human embryonic kidney (HEK-293) cells. Expression of HES1 and HEY1 both significantly down-regulate luciferase activity driven by the 1.0 kb (A) and 1.5 kb (B) *Gdnf* promoters. Results are given as mean $\pm$ standard error of the mean. ** $P < 0.01$ and *** $P < 0.005$.

FIG. 4. Levels of *Gdnf* expression in Sertoli cells inversely correlate to NOTCH activity in the seminiferous epithelium. Transgenic NOTCH Reporter mice (*TNR^{GFP/GFP}*) expressing GFP under the control of a promoter that requires NOTCH activation were crossed with dual transgenic mice expressing TdTomato specifically in Sertoli cells (*Amh-Cre; Rosa^{TdTomato/TdTomato}*) to yield the triple transgenic animal (*Amh-Cre; Rosa^{TdTomato/TdTomato}; TNR^{GFP/GFP}*). (A) Representative flow cytometry profile of labeled cells as isolated from (*Amh-Cre; Rosa^{TdTomato/TdTomato}; TNR^{GFP/GFP}*) mice along with the respective gates that were used to isolate dual labeled cells (TdTomato⁺ cells; Sertoli cells) of varying (low, medium, and high) levels of Notch activation (GFP expression). Cells of unknown identity (TdTomato⁻ cells) with or without GFP expression were isolated as well for comparison. (B). Quantitative RT-PCR analysis of cell populations. Levels of *Gdnf* expression inversely correlate to NOTCH activation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ indicate expression values significantly different from values obtained with Sertoli cells expressing low NOTCH activity. RT-PCR, real time PCR.

Germ cell presence is crucial to NOTCH signaling activation in Sertoli cells in vivo

Our previous work demonstrated that germ cells express the five NOTCH ligands (JAG1, JAG2, DLL1, DLL2, and DLL4) up to 100-fold the amount expressed by Sertoli cells [30]. To test whether NOTCH activation in Sertoli cells depends on the presence of germ cells, we treated our adult transgenic mice (*Amh-cre;Rbpj^{+/+};Rosa26^{YFP/YFP}* mice) with busulfan to induce loss of germ cells. Two months after treatment, Sertoli cells expressing YFP were isolated by FACS (Fig. 5A) and expression of NOTCH signaling targets

and *Gdnf* was quantified by qPCR. Data shown in Fig. 5B demonstrate a 4.2-fold increase in *Gdnf* expression by Sertoli cells after germ cell loss in comparison to Sertoli cells from mice with an intact germ cell compartment (FACS-sorted Sertoli cells from mice not treated with Busulfan). This increase in *Gdnf* expression by Sertoli cells confirms previous data obtained with W/Wv mice that are devoid of germ cells [22]. In addition, we observed a sharp decrease of the *Hey1* NOTCH target, while *Hes1* expression remained constant. These data demonstrate that NOTCH-induced GDNF downregulation in vivo might be controlled mainly by the transcriptional repressor HEY1.

FIG. 5. Ablation of germ cells in vivo results in decreased levels of NOTCH activation, and increased levels of *Gdnf*, in Sertoli cells. **(A)** Experiment schematic depicting busulfan treatment of *Amh-cre;Rosa^{YFP/YFP}* mice to ablate the germ cell compartment and FACS isolation of YFP⁺ cells to purify Sertoli cells. **(B)** Quantitative RT-PCR (qPCR) analysis. *Gdnf* is expressed by Sertoli cells and is required for spermatogonial stem cell maintenance; *Hes1*, *Hey1*, and *HeyL* are canonical Notch target genes, and *Dhh* is a marker of Sertoli cells. The increase in *Gdnf* levels in germ cell ablated mice may be due to absence of ligand expressing cells and a resulting decreased activation of NOTCH. **P* < 0.05 and ****P* < 0.005. FACS, fluorescence-activated cell sorting.

NOTCH activation and GDNF expression in Sertoli cells depends on spermatogonial JAG1 ligand

We next sought to broadly define which germ cells present the ligand(s) to the NOTCH receptor in Sertoli cells. To this end, we FACS-isolated specific subpopulations of germ cells (GFRA1⁺, KIT⁺, DAZL⁺KIT⁺, and DAZL⁺KIT⁻) corresponding to undifferentiated spermatogonia, differentiating spermatogonia and early spermatocytes. As seen in Fig. 6A, qPCR analysis revealed that undifferentiated spermatogonia highly express the *Jag1* ligand, with gradually fading expression as the germ cells differentiate. All other NOTCH ligands are weakly expressed in comparison (Fig. 6B–D). To investigate the influence of JAG1 on Sertoli cells in vitro, and to assess its effects on NOTCH target genes, we used primary cultures of WT Sertoli cells treated with and without immobilized JAG1. In some experiments, the cells were also transfected 1 day after ligand induction with dicer-substrate siRNA targeting *Hey1* or with scrambled siRNA (negative control). Our data show that activation of NOTCH signaling in cultured Sertoli cells, as measured by the induction of *Hey1* gene expression, depends on immobilized JAG1 (Fig. 7A). Importantly, ligand-

induced increase in *Hey1* gene expression was associated with significant decreases in *Gdnf* expression (Fig. 7B). Conversely, when *Hey1* transcripts were knocked down through siRNAs, the expression levels of *Gdnf* were significantly increased. Our results therefore demonstrate that *Hey1* gene expression in Sertoli cells is attributable to NOTCH pathway activation by JAG1, and that NOTCH signaling is modulating GDNF in vitro and in vivo.

Discussion

The NOTCH pathway is highly conserved and functions by juxtacrine cell–cell interactions in a wide variety of tissues. Its pleiotropic effects range from stem cell self-renewal, to fate specification and lineage maintenance, to somatic cell proliferation, apoptosis, and differentiation [58]. In response to ligand activation, the NOTCH receptor undergoes proteolysis to release NICD, which migrates to the nucleus to act as a transcription cofactor. In the canonical NOTCH signaling pathway, NICD associates with the DNA-binding protein RBPJ to stimulate transcription of downstream target genes, most notably *Hes* and *Hey* of the basic helix–loop–helix (bHLH) family of transcription

FIG. 6. JAG1 is mainly expressed by undifferentiated spermatogonia. Germ cells at different stages of differentiation were isolated from 6 to 10 dpp mice using sequential isolation by FACS. GFRA1⁺ germ cells are undifferentiated spermatogonia, KIT⁺ germ cells are differentiating spermatogonia, DAZL⁺KIT⁺ germ cells are type B spermatogonia/early spermatocytes, and DAZL⁺KIT⁻ germ cells are leptotene spermatocytes. *Jag1* is mainly expressed by undifferentiated spermatogonia, and expression decreases as germ cells mature (A). In comparison, *Jag2* (B) and *Dll4* (C) are expressed by differentiating spermatogonia and early spermatocytes, but expression is overall significantly weaker. The data suggest that JAG1 is the main ligand for NOTCH signaling in Sertoli cells. *Kit* expression in the different germ cell types is shown as comparison (D). In all graphs, gene expression data are all relative to expression in DAZL⁺KIT⁻ germ cells.

FIG. 7. JAG1-induced decreases in *Gdnf* expression in Sertoli cells is ablated through *Hey1* siRNA mediated knockdown. Freshly isolated Sertoli cells were cultured in the presence or absence of JAG1 before treatment with control (Scrambled) or *Hey1* siRNAs. **(A)** Quantitative RT-PCR (qPCR) analysis shows *Hey1* siRNA treatment effectively lowered *Hey1* expression levels in Sertoli cells treated with or without JAG1 ligand. **(B)** *Gdnf* expression is decreased in the presence of JAG1 ligand, however, knockdown of *Hey1* produces the opposite effect indicating ligand-induced regulation of *Gdnf* expression levels are mediated through *Hey1*. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$. siRNA, small interfering RNA.

factors (for reviews, see Iso et al. [32], Fischer and Gessler [59], Weber et al. [60]). Our previous studies demonstrated that in the testis, Sertoli cells lacking NOTCH signaling appeared normal but overexpressed the growth factor *Gdnf* [30], which is crucial for undifferentiated spermatogonia maintenance and proliferation [3,17]. Conversely, in Sertoli cells overexpressing NOTCH1, *Gdnf* is sharply down-regulated [29]. To demonstrate that modulation of GDNF expression is truly dependent on NOTCH signaling, we sought to show that (1) the NOTCH pathway is primarily canonical in Sertoli cells, (2) the targets of NOTCH HES1 and HEY1 truly bind the *Gdnf* promoter to downregulate its expression, and (3) NOTCH signaling is activated by germ cells expressing the JAG1 receptor.

Before this study, there was no in vivo evidence that the effects of NOTCH in the testis are mediated through RBPJ and its downstream targets HES1 and HEY1. Our rescue experiments using mouse models have therefore clarified this question. By rescuing the NOTCH1 overactivation phenotype that completely lacks germ cells [29] by knocking out RBPJ expression, we have now demonstrated that NOTCH1 strictly works through RBPJ and its downstream targets HES1 and HEY1. The testes still exhibited the phenotype observed in the *Rbpj* knockout model, presumably due to an absence of functional RBPJ at HES/HEY promoter binding sites, leading to reduced expression of these transcriptional repressors, and in turn effectively causing an excess of *Gdnf* expression. Furthermore, the fact that NOTCH1 overexpression had no effect on the RBPJ knockout phenotype demonstrates that NOTCH1 acts specifically on genes that require RBPJ for their expression. Our studies also demonstrate unequivocally that the HES1/HEY1 transcription factors directly bind to the *Gdnf* promoter to downregulate its expression, and that either molecule promotes this effect. Therefore, NOTCH signaling in Sertoli cells has the function of a suppressor of germ cell proliferation.

Further, our studies demonstrate that germ cell presence is necessary for NOTCH activation in Sertoli cells and that JAG1 is the predominantly expressed NOTCH ligand at the surface of germ cells. The preferential expression of JAG1 in GFRA1-positive germ cells, which are known to contain the stem cell pool, indicates that undifferentiated germ cells drive NOTCH signaling and their own homeostasis by down-

regulating *Gdnf* expression. However, it is important to acknowledge that activation of signaling pathways are dosage-dependent, therefore NOTCH activation might depend not only on the number of ligand molecules presented to the receptor by a particular germ cell type, but also on the number of germ cells presenting the ligand. In mouse models, JAG1 dosage is indeed crucial for the development and maintenance of other tissues such as the liver and heart [61,62]. In humans, *JAG1* loss-of-function mutations cause Alagille syndrome (AGS), an autosomal dominant disorder characterized by liver and heart insufficiency, vertebrae malformation, and retarded mental and sexual development [63,64]. In the testes of some male AGS patients (~33%), spermatogonia are present but do not differentiate [63], which might reflect an excess of GDNF expression. Further, as JAG1 and DLL may have opposite effects [65], future in vivo studies using ligand specific ablation in germ cells will answer the question of whether NOTCH signaling in Sertoli cells is activated/inactivated by a specific subset of ligands.

In summary, our results provide a molecular mechanism that modulates the expression and production of GDNF, a growth factor mainly found in the brain and testis that is crucial for the development and maintenance of dopaminergic and motor neurons, and undifferentiated male germ cells. We have demonstrated that NOTCH signaling in Sertoli cells of the testis is a crucial modulator of *Gdnf* expression through HES1 and HEY1 transcription factors, and that undifferentiated germ cells expressing the NOTCH ligand JAG1 regulate this system through negative feedback regulation.

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Author Disclosure Statement

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