Reproductive physiology of a humanized GnRH receptor mouse model: application in evaluation of human-specific analogs

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Tello JA, Kohout T, Pineda R, Maki R, Struthers RS, Millar RP. Reproductive physiology of a humanized GnRH receptor mouse model: application in evaluation of human-specific analogs. Am J Physiol Endocrinol Metab 305: E67–E77, 2013.—The human gonadotropin-releasing hormone receptor 1 (GNRHR1) is a 328-amino acid, seven transmembrane G protein-coupled receptor (GPCR) belonging to the class A rhodopsin-like receptors (25). It belongs to the mammalian type I GNRHRs that are the smallest of all GPCRs due to the lack of an intracellular COOH-terminal tail. In other GPCRs, the COOH-terminal tail is involved in receptor desensitization and internalization (25). The presence or absence of a COOH-terminal tail can influence the amount of receptor at the plasma membrane, as demonstrated by the fusion of the xenopus GnRHR tail to the human GNRHR1, which increased the quantity of cell surface receptors in both MCF7 (a breast cancer-derived cell line) and αT-4 cells (a gonadotrope-derived cell line) (34). Expression of the human GNRHR1 is low in heterologous cell lines compared with GNRHRs from other species due to a unique combination of structural features and amino acid differences (13, 34). For example, Lys191, specific to primate GnRH1, reduces expression by apparently destabilizing a cysteine bridge that enhances endoplasmic reticulum exit (20, 42). Glycosylation state also influences GnRHR expression, with rodent GnR1 being more highly expressed due to the presence of an additional NH2-terminal glycosylation site (Asn4) not present in the human GNRHR1. Addition of this extra glycosylation site to the human GNRHR1 increases the proportion of human receptor on the plasma membrane (8, 9). Inefficiently trafficked wild-type human GNRHR1 may be predisposed to other inhibitory effects such as mutations or intracellular influences that compound trafficking defects (1, 22, 42). The sensitivity of the cellular trafficking machinery to single amino acid differences can be illustrated by human GNRHR1 point mutations that can cause infertility due to intracellular retention of otherwise functionally competent receptors (3, 5, 25).

Several cell-permeant GnRH antagonists are able to restore receptor function by binding to the nascent receptor, thereby apparently stabilizing its folding and chaperoning the GNRHR1 protein through the ER to the plasma membrane (1, 19, 20). Interestingly, these pharmacological chaperones also modestly increase the amount of wild-type receptor at the cell surface (14). This is not unexpected, as only about one-half of the synthesized human receptors are estimated to be trafficked to the cell membrane in COS cells (1). The combination of these features results in the wild-type GNRHR1 with reduced recep-
tor at the plasma membrane, impaired agonist-dependent desensitization, and impaired receptor internalization compared with rodent and nonmammalian counterparts (4) that is not appropriately modeled in many laboratory animals.

Physiological studies focusing on the unique properties of the human GNRHR1 have been lacking, and only limited information has been garnered from the use of heterologous expression of the human GNRHR1 in derived cell lines, often not appropriately modeling the unique dynamics of the human GNRHR1 in vivo. To study the characteristics of the human GNRHR1 in an in vivo system, we established a mouse model in which the murine Gnrhr1 was substituted with the human sequence by germ line recombination. The complete human coding sequence was inserted into the first exon, resulting in a locus in which the human GNRHR1 is positioned under murine regulatory control. This “humanized” GNRHR1 mouse model provides a tractable in vivo experimental paradigm to assess the functioning of the human GNRHR1 in vivo and human-specific GNRHR1 therapeutics previously limited to primate testing.

Here, we present our findings on the impact of human GNRHR1 expression on various aspects of murine reproductive physiology and test the utility of this model to evaluate a human specific GnRH antagonist, NBI-42902.

MATERIALS AND METHODS

Animal Housing and Procedures

All mice were bred, housed, and tested in accordance with the United Kingdom Home Office Animals Scientific Procedures Act (1986) under Project License PPL 60/3843. The Home Office and the University of Edinburgh, under the consultation of a veterinary surgeon, approved all procedures. Mice were given food and water ad libitum and housed three to five per cage in 12:12-h light-dark cycles at 21°C.

Gene Targeting and Generation of Human GNRHR1-Ki Mice

The mouse chromosome 5 sequence was retrieved from the Ensembl database and used as a reference. The RP23-1653B3 BAC clone (C57BL/6J) was used for generating the homologous arms as well as the quantitative PCR (qPCR) probes for screening targeted events. The targeting vector used to generate the human GNRHR1 knock-in (ki) mice is shown in Fig. 1A. The human GNRHR1 cDNA along with the mouse 5′ homologous arm (2.2 kb) and 3′ homologous arm (3.7 kb) was generated by RED cloning/gap repair and confirmed by restriction digestion and end sequencing. The final targeting vector also contained fippase recognition target sequences flanking a neomycin expression cassette for positive selection in embryonic stem (ES) cells and a diptheria toxin A expression cassette for negative expression of ES cells. The NotI linearized targeting vector was electroporated into C57BL/6-derived mouse ES cells by Xenogen (Caliper Life Sciences, Alameda, CA). Positive ES cells were injected into blastocysts and transferred to pseudopregnant females, and germ line transmission of the knock-in allele was assessed by genomic DNA qPCR and confirmed by genotyping by using oligonucleotide primers (Eurofins MWG Operon) designed to regions spanning exon 1 of the mouse Gnrhr1 gene or the sequence encoding the neomycin resistance cassette in the wt or recombinant allele, respectively. Mice were rederived onto a C57BL/6/JolaHsd strain, which is homozygous for deletion of the α-synuclein and multimerin-1 genes (37, 38). The lines used in these experiments were of mixed status, and normal Mendelian ratios were obtained from heterozygote crosses.

Genomic Human GNRHR1 Transgene Detection by Genotyping PCR

Each genotyping PCR reaction contained 0.4 U Phusion DNA polymerase (Finnzymes, Finland), 1× Phusion high-fidelity PCR buffer, 0.2 mM deoxynucleoside triphosphate mixture (Invitrogen, Carlsbad, CA), 0.2 μM of each forward and reverse primer (Table 1), 13.2 μl of nuclease-free water, and 1 μl of template gDNA. PCRs were performed under the following conditions: initial denaturation at 98°C for 30 min, 40 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a 5-min final extension. The PCR amplicons were separated by electrophoresis on a 2% agarose gel.

Messenger RNA Isolation and cDNA Synthesis

Tissues were microdissected and rinsed in PBS before being snap-frozen in a microfuge tube. Each tissue was homogenized in ice-cold TRIzol reagent (Invitrogen) using a 5-mm stainless-steel bead and two cycles of 3 min at 25 Hz in a TissueLyser (Qiagen, Crawley, West Sussex, UK). Total ribonucleic acid (RNA) was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Each total RNA sample was then treated with ribonuclease-free deoxiribonuclease (Qiagen) and was purified using an RNeasy MinElute Clean-Up Kit (Qiagen). Total RNA (0.5 μg) was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen). To confirm DNA-free RNA, controls were run with all of the reaction components but lacked the reverse transcriptase enzyme (no RT).

Tissue Expression

Oligonucleotide primers (Eurofins MWG Operon) were designed to regions encoding exon 1 of human and mouse GNRHR1 genes. Each 20-μl reaction contained 0.4 U Phusion DNA polymerase (Finnzymes, Finland), 1× Phusion high-fidelity PCR buffer, 0.2 mM deoxynucleoside triphosphate mixture (Invitrogen), 0.2 μM of each forward and reverse primer (Table 1), 13.2 μl of nuclease-free water, and 1 μl of template cDNA. DNA amplification was performed under the following conditions: initial denaturation at 98°C for 2 min, 35 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a 5-min final extension. The amplicons were separated by electrophoresis on a 2% agarose gel. RT-PCR amplification of mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Table 1) was performed as an endogenous control for each tissue. Primer pairs that were specific for either mouse or human GNRHR1 were chosen, which was confirmed by the absence of amplicons after PCR amplification from cDNA made from either ki/ki or wt mouse pituitaries, respectively.

Real-Time Quantitative PCR

Serial dilution experiments were performed to optimize and verify quantitative reaction components; primer sets achieving at or above 95% efficiency were used in subsequent assays. Primers used to amplify mouse and human GNRHR1 were the same as those used for the tissue panel amplification. Each 20-μl PCR reaction contained 1× SYBR Green JumpStart Taq ReadyMix (Sigma), 1× ROX reference dye, 0.2 μM of each forward and reverse primer, 8 μl of nuclease-free water, and 1 μl of diluted cDNA. All reagents were mixed as a master mix and aliquoted into a 96-well PCR plate before 1 μl cDNA (diluted 6-fold with water) was added. Each sample was measured in triplicate, every PCR run included reaction controls without reverse transcriptase (no RT), and a cDNA pool was run on every plate as a calibrator. Amplification fidelity and sensitivity were verified by agarose gel electrophoresis and melting curve analysis. Real-time quantitative PCR was performed on an ABI Prism 7000 sequence detector system (Applied Biosystems) with an initial incubation at 95°C for 30 s, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for
30 s, during which fluorescence data were collected, ending with a melting curve analysis from 60 to 95°C. The expression of Gapdh was used as an endogenous control. The difference in threshold cycle (CT) between the assayed gene and Gapdh for any given sample was defined as $\Delta C_{T(X)}$. The difference in $\Delta C_{T(X)}$ between two samples was defined as $\Delta \Delta C_{T(X)}$, which represents a relative difference in expression of the assayed gene. The difference between the assayed gene relative to wt male Gnrhr1 expression was defined as $2^{-\Delta \Delta C_{T(X)}}$ (24).

Table 1. Primers used for RT-PCR and quantitative PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5’-3’)</th>
<th>Direction</th>
<th>Efficiency</th>
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<tr>
<td>Neo F</td>
<td>gccagctcatctctcactcactcact</td>
<td>Sense</td>
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<td>gctgatgtgtggta</td>
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<tr>
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<td>Sense</td>
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Mm, mouse; Hs, human; Gnrhr1, gonadotropin-releasing hormone receptor 1; Neo F, neomycin forward; App R, amyloid-β (A4) precursor protein reverse; App F, amyloid-β (A4) precursor protein forward.
Mouse Pituitary Membrane Binding

Mouse pituitaries were collected in ice-cold binding buffer consisting of 10 mM HEPES and 0.1% BSA, pH 7.4, supplemented with Complete Mini protein inhibitor tablets (Roche, UK). Three mouse pituitaries from each group were pooled and homogenized on ice in binding buffer by using 15 strokes of a Dounce tissue homogenizer. The homogenate was centrifuged at 500 g for 10 min at 4°C to remove nuclear debris and the lipid bilayer. The supernatant containing the crude membrane fraction was centrifuged twice at 70,000 g min at 4°C to remove nuclear debris and the lipid bilayer. The binding buffer by using 15 strokes of a Dounce tissue homogenizer.

Crude membrane homogenates (30 μg of protein) were incubated in triplicate with 85,000 counts/min (~0.2 nM) of [125I]-[D-Tyr6]-GnRH1 as radioligand with/without 1 μM nonradioactive GnRH1 in binding buffer (500 μl) in siliconized polystyrene microfuge tubes (Sigma-Aldrich) for 4 h at 4°C. After incubation, each suspension (450 μl) was transferred on top of ice-cold binding buffer containing 1.5% (wt/vol) bovine serum albumin (1 ml) in siliconized polystyrene microcentrifuge tubes and immediately centrifuged at 16,000 g for 10 min at 4°C. Supernatents were then aspirated, and the bottoms of the tubes were cut off, and radioactivity was assayed in a Wizard 1470 automatic γ-counter (PerkinElmer).

Hormone Measurement and GnRH Stimulation Test

All hormone measurements were conducted between 10 and 11 AM. Circulating serum LH, FSH, testosterone (T), estradiol (E2), and progesterone (P4) were measured in adult kiki and wild-type mice, with kiki and littermate females matched for the stage of estrous cycle (10–16 wk old). After a GnRH stimulation test, changes in circulating serum LH and T were measured; each mouse received an intraperitoneal (ip) injection of GnRH1 at a dose of either 1.25 or 125 ng/g body mass at time 0 with blood collection after 20 min. Blood was collected via cardiac exsanguinations of CO2 asphyxiated mice, and serum was separated by centrifugation at 2,000 g for 10 min. Serum was stored at −20°C until hormone assays.

Serum T levels were measured from male mice following solvent extraction, using a competitive radioimmunoassay as described previously (27, 28, 43). The limit of detection was 0.04 ng/ml, and the within-assay coefficient of variation was <10%.

E2 and P4 were measured from female mice following solvent extraction using the Ultra Sensitive Estradiol ELISA (DE4399) and Progestosterone ELISA (DE1561) assays (Demeditec, Kiel, Germany). Serum LH and FSH were measured using established in-house enzyme-linked immunosorbent assays as described (41). To assess the ability to specifically antagonize a GnRH-induced LH response in the kiki compared with wild-type mice, NBI-42902 (50 μg/g body mass) or vehicle (equal volume of 0.9% saline) was administered for 60 min, followed by a second ip injection of NBI-42902 (50 μg/g body mass) mixed with GnRH1 (1.25 ng/g body mass). Blood was collected 20 min after the second injection for serum LH measurement.

Puberty, Estrous Cycle, and Fertility Analysis

Beginning at 21 days of age, females were examined daily for vaginal opening as an indirect indicator of puberty onset. Following vaginal opening, vaginal smears were taken at the same time daily by gently performing vaginal lavages with water and transferring cells to a microscope slide. Vaginal lavages were taken daily (10 AM) for a period of 15 days to assess the estrous cycles of kiki and wt females (6–8 wk of age). Cells were dried, fixed in methanol, and stained using the Papanicolaou technique. Estrous cycle staging was assessed under a microscope, where proestrous was assigned as predominantly basal and nucleated cells, estrous was assigned as predominantly cornified epithelial cells, metestrous was assigned as mixed cornified epithelial cells and leukocytes, and diestrous was assigned as predominantly leukocytes.

To examine the possible effects of genotype on fertility, adult female mice (16 wk old) were fixed in 10% formalin solution and stored at 4°C. Tissues were dehydrated through a graded series of ethanol to 100% and then embedded in paraffin wax. Embedded gonads were sectioned at 5-μm thickness, deparaffinized using xylene, and rehydrated through decreasing series of alcohol in water. The hydrated sections were stained with hematoxylin and eosin, examined, and photographed on a Zeiss microscope.

Statistical Analysis

Data were analyzed by a two-tailed unpaired Student’s t-test (between 2 groups) or by using one-way ANOVA followed by Tukey’s multiple comparison test (between more than 2 groups), where P < 0.05 was considered to be statistically significant. Hormone values below detection were assigned the lowest value on the standard curve.

RESULTS

Human GNRHR1 Gene Insertion in Mice

Crossing germline chimeras with C57BL/6 wt mice produced heterozygous mice for the targeted allele C57BL6J-human GNRHR1-knock-in, hereafter designated ki. PCR sequencing confirmed that the targeting vector containing the human GNRHR1 coding sequence was inserted into exon 1 of the mouse Gnrhr1 gene (Fig. 1A). Quantitative PCR analysis of genomic DNA confirmed that mutant mice carried only a single targeted insertion compared with the single copy gene encoding the mouse amyloid β-precursor protein (App; 1 copy and 0.5 copy/App for kiki and +/ki, respectively; Fig. 1B). Genomic DNA tested with a probe to the neomycin resistance gene generated DNA amplicons of the expected size in the homozygous and heterozygous mutant mice and was absent in wt genomic DNA as expected (Fig. 1C). Genotyping of wild-type (+/+), heterozygous (+/ki), and homozygous (kiki) mice with the primers for RT-PCR resulted in a ki amplicon of 573 bp and a wt amplicon of 340 bp (Fig. 1C). Homozygous humanized GNRHR1-ki (kiki) mice born to heterozygous parents were viable and had a genotype ratio of 52:81:54 (wild type/heterozygous/homozygous).

Characterization of Humanized GNRHR1 Knock-In Mice

Human Gnrhr1 expression. Mouse Gnrhr1 transcripts were amplified exclusively from cDNA pools made from the
pituitary and gonads from wild-type mice (+/+), whereas homozygous (ki/ki) mice had human GNRHR1 expression predominantly in the pituitary and gonads but also a low level of expression in other tissues, including muscle, liver, heart, cortex, hypothalamus, and the olfactory bulb (Fig. 1D).

**Pituitary GNRHR1 mRNA and protein have reduced expression in ki/ki mice.** Quantitative PCR detected significantly reduced human GNRHR1 transcripts in the pituitary from ki/ki mice compared with mouse Gnrhr1 transcripts in wild-type littermates [ki/ki males were 6.8-fold less (n = 4), and ki/ki females (n = 4) were 7.8-fold less than their wt littermates; Fig. 2A]. Corroborating this low mRNA expression level, the quantity of bound radiolabeled GnRH1 from pituitary membrane fractions was drastically reduced in ki/ki mice compared with wild type [maximal binding was 4,660 ± 379 counts/min, n = 9 (ki/ki), compared with 9,082 ± 693, n = 9 (wt); Fig. 2B], indicating a decreased level of GNRHR1 protein at the membrane. Although reduced levels of GNRHR1 transcripts and GNRHR1 protein were found, no clear differences in gonadotrope morphology were detected when assayed for LH or FSH immunoreactivity (Fig. 2, A and C).

**Normal puberty but reduced fertility in female humanized GNRHR1 knock-in mice.** Homozygous (ki/ki) females were externally indistinguishable from wild-type littermates with a similar onset of vaginal opening [31.4 ± 1.4 days, n = 10 (ki/ki), vs. 30.9 ± 0.7 days, n = 8 (wt)] and day of first estrus [37.0 ± 1.3 days, n = 9 (ki/ki), vs. 37.5 ± 1.1 days, n = 8 (wt); Fig. 3A]. The fertility of female ki/ki mice compared with wild-type littermates was assessed after pairing with proven fertile wild-type males for 90 days. Female ki/ki mice had a significantly reduced number of litters compared with wild-type littermates [2.0 ± 0.2, n = 7 (ki/ki), vs. 3.2 ± 0.2, n = 6 (wt), P < 0.0017; Fig. 3B], with no difference in litter size [7.2 ± 0.6, n = 13 (ki/ki), vs. 6.2 ± 0.5, n = 18; Fig. 3C]. The time to first litter was not different between ki/ki and wild-type females [25.2 ± 5.0 days, n = 6 (ki/ki) vs. 22.8 ± 1.4 days, n = 6 (wt); Fig. 3D] but ki/ki females had significantly longer periods between litters [33.3 ± 3.2 days, n = 6 (ki/ki), vs. 26.2 ± 1.9 days, n = 12 (wt), P < 0.0281; Fig. 3E]. In addition, female ki/ki mice displayed high variability in the length of each estrous stage, with some animals having periods of prolonged vaginal cornification, leading to lengthened stages of estrous and/or metestrous (Fig. 3F). Adult uterine and ovarian weights in ki/ki mice were not significantly different from wild-type littermates [uterine weight (mg): 80.5 ± 10.0, n = 9 (ki/ki), vs. 80.1 ± 5.5, n = 9 (wt) (Fig. 4A); ovarian weight (mg): 2.7 ± 0.3, n = 8 (ki/ki), vs. 3.5 ± 0.3, n = 9 (wt) (Fig. 4B)], nor was the gross morphology of the ovaries different between genotypes (Fig. 4C).

**Male GNRHR1 knock-in mice have normal testicular development.** Homozygous (ki/ki) males were indistinguishable from wt littermates with normal anal-genital distance and normal genital papilla. The weight of adult ki/ki testes was not significantly different from wild-type littermates [testes weight (mg): 96.2 ± 3.2 (ki/ki) vs. 101.6 ± 8.1 (wt); Fig. 4D], and the morphology of the testes showed seminiferous tubules with all

**Fig. 2. Pituitary expression of human and mouse GnRHRs and immunolocalization of gonadotropins.** A: relative GNRHR1 mRNA level assessed by SYBR Green real-time PCR assay for control (n = 4) and ki/ki mice (n = 4) expressed as fold change ± SE of normalized GNRHR1 mRNA levels relative to male wild-type Gnrhr1. B: competitive binding assay of [125I]I-Trp6-GnRH1 on crude pituitary membrane fractions made from female wild-type and humanized GNRHR1-knock-in (ki/ki) mice. C and D: representative images showing the immunolocalization of LH (C) and FSH (D) in the pituitaries from wild-type and ki/ki mice. Pituitaries were sectioned at 5 μm. Magnification is shown at left. Scale bar, 50 μm. NS, not significant, *P > 0.05, **P < 0.05, ***P < 0.001, and ****P < 0.0001, statistical difference in an unpaired t-test.
stages of germ cell development. Leydig cells were present in the interstitial tissue, and well-formed Sertoli cells appeared mature (Fig. 4E).

**Human GNRHR1 knock-in mice have altered responses to GnRH challenge.** Basal serum gonadotropin hormones (LH and FSH) in both male and female ki/ki mice were not significantly different compared with wild-type littermates (Fig. 5, A and B). However, responses to GnRH challenge resulted in a significantly reduced LH stimulation in both male and female ki/ki mice compared with their wild-type littermates [1.25 ng/g GnRH1 dose: males, 5.5 ± 0.9 ng/ml, n = 7 (ki/ki), vs. 8.8 ± 0.4 ng/ml, n = 5 (wt), P < 0.0058; females, 1.5 ± 0.4 ng/ml, n = 6 (ki/ki), vs. 3.9 ± 0.4 ng/ml, n = 6 (wt), P < 0.0014; 125 ng/g GnRH1 dose: males, 5.6 ± 0.4 ng/ml, n = 5 (ki/ki), vs. 7.0 ± 0.3 ng/ml, n = 4 (wt), P < 0.0188; females, 6.3 ± 0.5 ng/ml, n = 4 (ki/ki), vs. 7.3 ± 0.2 ng/ml, n = 5 (wt), P < 0.0334]. Likewise, levels of serum testosterone did not significantly differ between ki/ki and wt males (Fig. 5C), but the testosterone response to a GnRH challenge was reduced in ki/ki males [5.5 ± 1.2 ng/ml, n = 5 (ki/ki), vs. 15.7 ± 5.0 ng/ml, n = 5 (wt), P < 0.0403]. In female mice, basal circulating serum progesterone did not differ, but basal serum estradiol was significantly higher in ki/ki mice compared with estrous stage-matched wt littermates [13.6 ± 2.9, n = 8 (ki/ki), vs. 3.0 ± 0.8, n = 10 (wt), P < 0.0055; Fig. 5D]. Basal levels of LH and T were no different from control injected mice; therefore, data from vehicle injections are included in Fig. 5, A and C.

Characterizing properties of a human-specific GnRH analog in the humanized mouse model. To evaluate our humanized GNRHR1-ki mice as a useful pharmacological model, we assessed the ability of a human-specific GNRHR1 antagonist, NBI-42902, to suppress a GnRH-induced LH rise in homozygous (ki/ki) male mice compared with their wild-type littermates. Levels of serum LH after vehicle administration were similar in homozygous (ki/ki) male mice and wild-type controls [0.2 ± 0.1 ng/ml, n = 6 (ki/ki), vs. 0.3 ± 0.1 ng/ml, n = 5 (wt), respectively]. Both genotypes displayed a robust GnRH1-induced serum LH response [6.6 ± 0.7 ng/ml, n = 5 (ki/ki), vs. 8.8 ± 0.4 ng/ml, n = 5 (wt)]. Whereas treatment with NBI-42902 abrogated the GnRH-induced rise in serum LH only in (ki/ki) mice [vehicle: 0.2 ± 0.1 ng/ml, n = 6; NBI + GnRH1:
0.5 ± 0.1 ng/ml, n = 6; GnRH1: 6.6 ± 0.7 ng/ml, n = 5), with little effect on wild-type littersmates [vehicle: 0.4 ± 0.1 ng/ml, n = 5; NBI + GnRH1: 6.4 ± 0.4 ng/ml, n = 6; GnRH1: 8.8 ± 0.4 ng/ml, n = 5; Fig. 6].

Impaired negative feedback after GDX in humanized mice. To evaluate the effect of gonadal hormone feedback upon higher levels of the hypothalamic-pituitary-gonadal axis in kilki mice, we monitored circulating LH levels after GDX in adult male mice. Interestingly, kilki mice did not display a post-GDX increase in serum LH even after 3 wk of LH monitoring, whereas wt littersmates exhibited robust LH elevations during the 3 wk following GDX [0.3 ± 0.1 (intact), 0.4 ± 0.1 (1 wk post-GDX), 0.3 ± 0.1 (2 wk post-GDX), and 0.6 ± 0.1 ng/ml (3 wk post-GDX), n = 6 (kilki), vs. 0.2 ± 0.1 (intact), 6.5 ± 1.2 (1 wk post-GDX), 5.9 ± 0.6 (2 wk post-GDX), and 5.7 ± 0.5 ng/ml (3 wk post-GDX), n = 6 (wt); Fig. 7].

DISCUSSION

It has long been known that the density of GnRH receptors on gonadotrope membranes determines the ability of the pituitary to respond to GnRH (45). The quantity of a receptor on the cell membrane is determined by a multitude of factors, including gene expression, mRNA stability, nascent protein synthesis, posttranslational processing and glycosylation, protein trafficking, protein stability, and protein recycling/degradation. Using homologous recombination, we targeted the insertion of the coding sequence of the human GNRHR1 to the first exon of the mouse Gnrh1 gene to exploit the native transcriptional machinery of the mouse Gnrh1 gene. Despite this, analysis of kilki pituitaries revealed a significantly reduced transcript level of human GNRHR1 compared with mouse Gnrh1 in wild-type littersmates (~7- to 8-fold less), which is similar to the difference in expression found with transfection of the human and murine receptors in many cell lines. These differences in expression reveal an alteration of the murine transcriptional control regulating the human GNRHR1 transgene and/or instability of the human GNRHR mRNA transcript. Unexpectedly, we found two partial mouse Gnrh1 transcripts expressed in kilki pituitaries. Sequencing identified these as abnormally spliced transcripts encoding part of the 3' end of neomycin resistance cassette ending with a stop codon linked to exons 2 and 3 of the mouse Gnrh1. We do not predict translation of these partial mouse Gnrh1 transcripts due to the upstream stop codon and a lack of a Kozak sequence but are unaware of how this may impact transcription of the human GNRHR1 transgene.

A complex enhancer consisting of steroidogenic factor-1 and activator protein-1 binding sites and a region called the GnRH receptor-activating sequence have been found to regulate the basal cell-specific activity of the murine Gnrh1 (11, 12) as well as increase Gnrh1 expression mediated by multiple endocrine
inputs (10, 44). This enhancer should not have been affected by the downstream insertion of the transgene, but the spatial arrangement with other unknown regulatory elements (e.g., downstream enhancers) may have been disrupted by the transgene insertion. Interestingly, the insertion of the transgene not only appears to have influenced the functioning of transcriptional enhancers but may have also affected tissue-specific repressor elements, as evidenced by the broad expression of the human GNRHR in tissues that do not express wt mouse Gnrhr1. Evidence for a Gnrhr transcriptional repressor was identified recently, where elimination of a neural-restrictive silencer factor binding site in the 5′ promoter of the rat Gnrhr increased luciferase activity more than 10-fold in vitro (31). In accord with the reduced pituitary transcript levels of the human GnRH receptor, we found significantly lower radiolabeled GnRH binding in pituitary membrane fractions prepared from female kilki mice compared with their wild-type counterparts, indicative of this lower GNRHR protein found in the kilki mice. Properties inherent to the human GNRHR1 may have contributed to this lower level of protein expression within the cell membrane homogenates. As mentioned previously, the human GNRHR1 may have predisposed to misfolding and intracellular retention due to a unique set of properties, including the apparent disruption of a sulfhydryl bond by a primate-specific Lys191 (17), diminished targeting to the cell membrane by the absence of an intracellular COOH-terminal tail, and reduced protein stability due to the reduction in glycosylation sites to one compared with two in rodent counterparts (9, 33). Because these properties are found in some other primates, it has been suggested that this is a recently evolved mechanism to facilitate rapid posttranslational regulation of gonadotrope cell surface numbers (18). The pool of intracellularly retained GnRH receptors is thus postulated to provide a reservoir that can be rapidly recruited to the cell without the need for changes in transcription or translation. However, there is no direct experimental evidence for this proposition. The reduced numbers of GNRHR1 resident on the gonadotrope membrane in kilki mice appear to be the result of both decreased transcriptional efficiency and inefficient trafficking to the membrane. Although we do not know whether or not this faithfully mimics the mechanisms underlying reduced receptor cell surface expression in humans, it does provide a model of reduced expression of the human GNRHR1 in vivo for studying the utility of GnRH pharmacochaperones for the rescue of cell surface expression in the human receptor. This is relevant in view of susceptibility of the human GNRHR1 to intracellular retention resulting from GNRHR1 missense mutations that cause hypogonadotropic hypogonadism due to misrouting of an otherwise functional receptor (3).

Female Puberty and Ovarian Cyclicity

Although female kilki mice exhibited severely reduced pituitary levels of GnRHR, they displayed normal pubertal development with a similar day of vaginal opening compared with wt littermates and a normal time to first estrous. Vaginal opening occurs in response to rising levels of circulating estrogen levels after the emergence of pulsatile GnRH secretion during puberty (21). Only a few GnRH neurons are necessary to initiate pulsatile LH secretion (15), and no more than ~70 GnRH neurons (only ~12% of total) are needed to promote normal puberty (16). However, larger numbers are
required for more complex cyclical functions of the gonadotropin axis, including the preovulatory LH surge (16). These data indicate that low levels of GnRH signaling in the form of low GnRH secretion in those studies, or in the form of reduced cell surface receptor in our studies, can still promote pubertal maturation but are insufficient for full adult reproductive function. Interestingly, evaluation of estrous cycling showed that ki/ki mice exhibited prolonged periods of estrous or metestrous, with some ki/ki mice displaying persistent vaginal cornification. The fertility of female ki/ki mice was reduced significantly, with longer periods between litters compared with wt females. Surprisingly, circulating levels of pituitary gonadotropins were normal, but females displayed elevated serum E2 levels compared with wt littermates. High E2 levels and irregular cycles are representative of middle-aged acyclic rodents with a corresponding loss of reproductive cycles (30).

In addition, administration of E2 to wt adult mice can cause a rapid onset of persistent vaginal cornification characteristic of reproductive senescence (29). To test the possibility that ki/ki females were susceptible to premature age-related acyclicity and senescence, we repeated the 15-day estrous cycle evaluation on the same ki/ki mice after 24 wk, and the cycles were no worse (data not shown). Although the high levels of estradiol observed in ki/ki mice can explain the irregular estrous cycles, we are unsure how this occurs with reduced pituitary GnRHR1 expression and normal serum LH. The elevated serum E2 levels did not appear to affect the gross morphology of the ovary, as female ki/ki mice had normal ovarian weights and the ovaries revealed no obvious histological abnormalities. Interestingly, these mice could be crossed with other genetic models with loss-of-function or reduced function in other reprod-

**Male Reproductive Physiology**

Male ki/ki mice displayed normal reproductive maturation, testicular development, and circulating gonadotropins and testosterone levels, with no deficits in fertility when paired with wild-type females (data not shown). Despite this, they exhibited impairment in negative feedback by gonadal sex steroids at higher levels of the hypothalamic-pituitary-gonadal axis, as male ki/ki mice did not display increased serum LH after gonadectomy even after 3 wk of LH monitoring. Negative feedback by gonadal hormones can occur at the level of both the hypothalamus and the pituitary. A recent study clearly demonstrated the impact of removing the pituitary’s role in gonadal steroid negative feedback; female mice with pituitary-specific ERα knockout displayed elevated nonsurge serum LH and E2 levels, leading to either subfertility or complete infertility (35). In addition, gonadectomized ERα knockout females replaced with E2 were unable to suppress LH to levels found in wild-type littersmates.

**Response to Exogenous GnRH**

The high secretory rate of LH that is necessary for ovulation reflects both enhanced GnRH secretion and increased pituitary

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Fig. 6. Specific antagonism of the GnRH-induced LH surge in humanized ki/ki mice by NBI-42902. Serum LH in adult male mice after an initial intraperitoneal injection of NBI-42902 or vehicle, followed by a second injection of NBI-42902 (50 ng/g body wt) combined with GnRH1 (1.25 ng/g body wt) after 60 min. Blood was collected 20 min after the second injection via cardiac exsanguinations of CO2-asphyxiated mice. Data are means ± SE. ***p < 0.001, statistical difference as measured by 1-way ANOVA followed by Tukey’s multiple comparison test.

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Fig. 7. Effect of gonadectomy (GDX) on serum LH levels in adult male mice. GDX was performed on adult male mice (3 mo old), and circulating LH was monitored for 3 wk following GDX. Blood was collected via submandibular puncture. Data are means ± SE. **p < 0.01 and ***p < 0.001, statistical difference as measured by 1-way ANOVA followed by Tukey’s multiple comparison test.
responsiveness to GnRH. The pituitary becomes sensitized to GnRH by increasing the expression of GnRH receptors in gonadotropes that respond to GnRH stimulation (36). Both female and male ki/ki mice had significantly fewer receptors on the cell membrane, suggesting that they would have a lower signaling capacity. This was borne out by GnRH challenge, where ki/ki mice had significantly reduced serum LH response to both low-dose (1.25 ng/g) and high-dose (125 ng/g) GnRH challenge compared with wild-type littermates. The large dose of GnRH was chosen to exclude the chance of extrapituitary GNRHR1 sites present in the ki/ki mice, effectively reducing pituitary exposure to GnRH. These findings indicate that features of the reproductive axis that require relatively simple pulsatile GnRH signaling are not impacted by reduced numbers of GNRHR at the plasma membrane. In contrast, more complex cyclical elements required for normal estrous cycles may have been impacted.

Although reduced human GNRHR1 was present on pituitary membrane fractions, it did not affect gross pituitary morphology or the numbers of LH/FSH-immunoreactive gonadotropes compared with wt littermates, and it appears that circulating gonadotropin levels were no different in males and females.

Screening Human-Specific GnRH Analogs

GnRH analog suppression of sex steroid hormones is utilized in the treatment of many hormone-dependent disorders of the reproductive tract, including cancers of the prostate, ovary, and breast by suppression of sex steroid-induced growth (32, 40). They are also employed extensively in assisted reproductive therapy to prevent ovulation and better control ovarian hyperstimulation, in precocious puberty to delay pubertal onset (2), and in children with gender incongruency/dysphoria to delay puberty, pending treatment decisions (23). Recently, preclinical studies have demonstrated rescue of cell surface trafficking defective GNRH receptor mutants that cause hypogonadotropic hypogonadism (4, 6).

To assess the utility of our mouse model for the development of GnRH antagonists specific for the human GNRHR1, we measured the ability of NBI-42902 to antagonize specifically the human GNRHR1 in ki/ki compared with wild-type littermates. The abrogation of the GnRH-induced LH serum response in ki/ki mice but not in littermates with the murine GnRH receptor highlights the potential of our mouse line as a novel “humanized” model for human-specific receptor targeting therapeutics/interventions. The model will thus allow the direct assessment of human-specific GnRH analogs in vivo. For example, GnRH peptide analogs are the prime treatment for prostate cancer to selectively reduce testosterone production. This mouse model might be utilized to monitor effective testosterone inhibition by novel small-molecule GnRH antagonists such as NBI-42902. Similarly, the uses of orally active small-molecule GnRH antagonists are proposed for use in women’s health targets such as endometriosis and polycystic ovary syndrome to partially suppress estrogen production. Combining our mouse model with murine models of endometriosis and polycystic ovarian syndrome could assess the efficacy of these analogs in these diseases.

Summary

We report the generation and characterization of a humanized mouse model by disruption of the mouse Gnrhr1 gene locus with the insertion of the human GNRHR1 coding sequence using homologous recombination. Pituitary GNRHR1 transcripts and functional protein are lower in ki/ki mice compared with their wt counterparts in both sexes. Assessment of the reproductive physiology of male mice revealed that ki/ki mice achieve normal reproductive benchmarks and have normal circulating serum gonadotropin levels. In addition, males have normal circulating testosterone levels and achieve mature testicular development with normal testicular weights. Females display normal levels of serum progesterone but display elevated estradiol. Female reproductive organs from ki/ki mice appear to develop normally, achieving normal adult uterine and ovarian weights. However, ki/ki females exhibit erratic estrous cycles and reduced fertility. Challenging ki/ki mice with ip administration of GnRH resulted in reduced serum LH responses in ki/ki mice of both sexes in addition to a reduced testosterone response in males. Interestingly, gonadectomy of ki/ki males did not result in raised serum LH even after 3 wk, indicating a disruption of the negative feedback mechanism of gonadal steroids on higher levels of the hypothalamic-pituitary-gonadal axis. After consideration of the altered female reproductive physiology, our model can be used to assess various human-specific GnRH targeting paradigms, as shown by our specific abrogation of a GnRH challenge in ki/ki mice but not in wild-type controls.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.A.T., T.K., R.A.M., R.S.S., and R.P.M. contributed to the conception and design of the research; J.A.T., T.K., R.P., and R.A.M. performed the experiments; J.A.T., T.K., R.A.M., and R.P.M. analyzed the data; J.A.T., T.K., R.S.S., and R.P.M. interpreted the results of the experiments; J.A.T. prepared the figures; J.A.T. drafted the manuscript; J.A.T., R.P., and R.P.M. edited and revised the manuscript; J.A.T. and R.P.M. approved the final version of the manuscript.

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