

Human Relaxin Receptor Is Fully Functional in Humanized Mice and Is Activated by Small Molecule Agonist ML290

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Relaxin, a small peptide hormone of the insulin/relaxin family, demonstrated antifibrotic, organ protective, vasodilatory, and proangiogenic properties in clinical trials and several animal models of human diseases. Relaxin family peptide receptor 1 (RXFP1) is the relaxin cognate G protein-coupled receptor. We have identified a series of small molecule agonists of human RXFP1. The lead compound ML290 demonstrated preferred absorption, distribution, metabolism, and excretion profiles, is easy to synthesize, and has high stability *in vivo*. However, ML290 does not activate rodent RXFP1s and therefore cannot be tested in common preclinical animal models. Here we describe the production and analysis of a mouse transgenic model, a knock-out/knock-in of the human *RXFP1* (*hRXFP1*) complementary DNA into the mouse *Rxfp1* (*mRxfp1*) gene. Insertion of the vector into the *mRxfp1* locus caused disruption of *mRxfp1* and expression of *hRXFP1*. The transcriptional expression pattern of the *hRXFP1* allele was similar to *mRxfp1*. Female mice homozygous for *hRXFP1* showed relaxation of the pubic symphysis at parturition and normal development of mammary nipples and vaginal epithelium, indicating full complementation of *mRxfp1* gene ablation. Intravenous injection of relaxin led to an increase in heart rate in humanized and wild-type females but not in *Rxfp1*-deficient mice, whereas ML290 increased heart rate in humanized but not wild-type animals, suggesting specific target engagement by ML290. Moreover, intraperitoneal injection of ML290 caused a decrease in blood osmolality. Taken together, our data show humanized RXFP1 mice can be used for testing relaxin receptor modulators in various preclinical studies.

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Relaxin peptide (RLN) is a member of the insulin/relaxin family, produced mainly in female and male reproductive organs [1]. RLN cellular signaling is mediated by its cognate G protein-coupled receptor RXFP1, relaxin family peptide receptor 1 [2]. Initially, RLN was discovered and named for its relaxation/elongation effect on the pubic symphysis during parturition in guinea pigs, which facilitates delivery of pups. Injection of RLN also resulted in a substantial decrease of myometrial contractions and uterine quiescence. Subsequently, potential therapeutic

Abbreviations: ANOVA, analysis of variance; cDNA, complementary DNA; ES, embryonic stem; HR, heart rate; IHC, immunohistochemistry; IRES, internal ribosome entry site; LacZ, beta-galactosidase gene; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RLN, relaxin peptide; RT-PCR, reverse transcription polymerase chain reaction.

properties of this peptide were discovered, such as its beneficial effects on collagen decrease in various rodent fibrotic models [3]. It was also shown that RLN has potent vasodilatory properties and is involved in renal compliance during pregnancy [4].

Analysis of the phenotype of *Rln1*- or *Rxfp1*-deficient mice revealed several abnormal features [5–8]. Some mutant females were unable to deliver pups because of failure of the pubic symphysis to dilate. Mutant females also had undeveloped mammary nipples and were unable to feed pups. Analysis of the pregnant female reproductive tract showed poor differentiation of the vaginal and cervical epithelium. Age- and sex-dependent fibrosis developed in several mutant organs, with a progressive increase in collagen content [3]. These data motivated further testing of RLN as a therapeutic agent in various preclinical models of human diseases, including models of liver, kidney, heart, and lung fibrosis; pulmonary hypertension; allergic airways disease; type 1 diabetes; and other acute and chronic conditions [2].

Recombinant human relaxin 2 peptide (serelaxin) has been tested as a therapeutic agent in several clinical trials, including systemic sclerosis, induction of labor, and heart failure [9]. These results suggested that RXFP1 can be a therapeutic target. However, the use of RLN as a drug requires its continuous intravenous delivery because of low *in vivo* stability. The mode of administration and the cost of production make serelaxin treatment expensive and difficult for use in chronic diseases. A small molecule RXFP1 agonist with the potential for oral delivery would be the preferred option [10].

ML290 is the first small molecule RXFP1 agonist demonstrating RLN-like activity at cellular levels, receptor specificity, *in vitro* and *in vivo* stability, low cytotoxicity, and an excellent absorption, distribution, metabolism, and excretion profile [11]. However, unlike human RLN, ML290 and other compounds from this series do not activate rodent RXFP1 to induce cyclic adenosine monophosphate production in cells transfected with the receptor [12]. In fact, ML290 behaved as a partial inverse allosteric agonist in this assay, suppressing RLN-induced activation of the mouse receptor [13]. Therefore, various well-established genetic and induced mouse models of human diseases cannot be used to test ML290's therapeutic effects. The obvious solution is the production of an RXFP1 humanized transgenic model. However, the question arises whether human and mouse RXFP1s have similar activation ability and whether downstream cell signaling pathways and physiologic responses induced by human GPCR will be the same in mice.

Here we describe the production and characterization of humanized mice expressing human *RXFP1* instead of the mouse receptor. The internal ribosome entry site (IRES)-driven complementary DNA (cDNA) of *hRXFP1* was inserted into one of the mouse exons, resulting in ablation of endogenous gene expression but production of the functional human receptor. *hRXFP1* complemented mouse *Rxfp1* gene ablation in humanized females, which presented the wild-type phenotype. Furthermore, the effects of ML290 and RLN on heart rate (HR) showed that humanized mice respond to both ligands, whereas wild-type mice do not respond to ML290. Target engagement by the native hormone and small molecule agonist in humanized *RXFP1* mice provides an opportunity to test the therapeutic effects of new hRXFP1 modulators in various genetic and experimentally induced mouse models of human diseases.

1. Materials and Methods

A. Generation of Humanized Mice

All animal studies were approved by the Florida International University Animal Care and Use Committees in accordance with the principles and procedures outlined in the National Research Council Publication Guide for Care and Use of Laboratory Research Involving Animals under Protocols 14-016 and 16-003. The embryonic stem (ES) cell targeting experiments and the ES cell microinjections into blastocysts were performed in the Mouse ES Cell Core and Genetically Engineered Mouse Core at Baylor College of Medicine, Houston, Texas.

Humanized mice were created using ES cell targeting technology by the approach described previously for knock-out/beta-galactosidase gene (*LacZ*) knock-in recombinant allele into the mouse *Rxfp1* locus [6]. The same insertional targeting construct was used in this project, except *LacZ* cDNA was substituted with the full-length human *RXFP1* cDNA encoding an open reading frame of the gene and the bovine growth hormone polyA signal from pCR3.1 vector (Invitrogen, Carlsbad, CA) (Fig. 1). The various fragments of the targeting construct were polymerase chain reaction (PCR) amplified from corresponding plasmids and cloned together using In-Fusion Cloning kit (Clontech Laboratories, Mountain View, CA). The final targeting construct was sequenced to ensure there were no errors during the PCR amplification or cloning. The targeting vector was linearized with *PshAI* restriction site located inside the mouse genomic fragment and electroporated into JM8 ES (C57BL/6N) cells. Selection of recombinant clones was performed with G418 (Geneticin). Three hundred ES cell clones were screened by long-range PCR using Advantage PCR polymerase (Clontech Laboratories) and vector-specific primers (IRES2R, TGGCTGCAGGTCGACAAGAT and K1scr3endF, GGGCTGCAGGAATTCGATAT) and primers from mouse *Rxfp1* exons 15 and 17 (mRxfp1ex15F-ki, CCAACACGGATGGGATTTCA and mLgrEx17R, CCACCTGAAGCAGTGAAAGA) located outside the genomic DNA used for vector construction. The recombinant ES clone was used for microinjections into blastocysts and implanted into pseudopregnant females. Chimeric males were bred with C57BL/6 females. Presence of the humanized allele was detected by PCR of ear DNA with human-specific primers hLGR7ex1/2bpF, TGACATCTGGTTCTGTCTTCTTCT, and hLGR7ex2/158bpR, CAGTCGTCCACACCGTTACA. Mice with the knockout allele of *Rxfp1*, *Rxfp1-LacZ*, were also used in this study, and the presence of this allele was detected with *LacZ*-specific primers as described in the original publication [8]. All mice used in this study were on the C57BL/6 background.

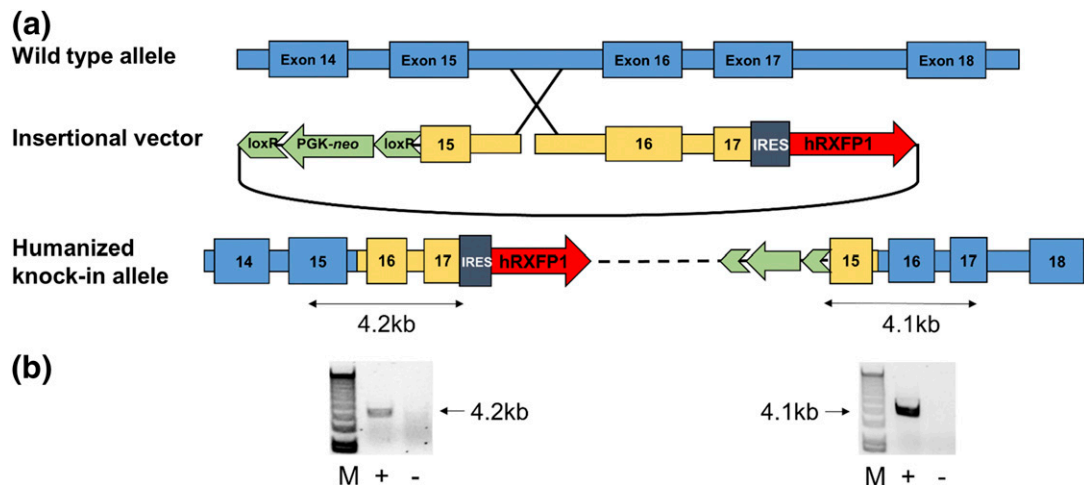


Figure 1. Schematic representation of *hRXFP1* knock-in targeting. (a) Human *RXFP1* cDNA was introduced by homologous insertion of the targeting vector into mouse *Rxfp1* locus in ES cells (top). Insertional targeting construct (middle) was prepared by insertion of the 4-kb mouse *Rxfp1* genomic fragment and the IRES–full-length *hRXFP1* cDNA construct into Bluescript II KS (–) vector with floxed neomycin resistance cassette. Targeting vector was linearized with *PshAI* restriction enzyme cutting within the genomic fragment. After homologous integration (bottom) of the targeting construct, the backbone of the targeting vector was inserted into the chromosome. A genomic fragment in the targeting vector was duplicated (part of exons 15 and 17, and exon 16). The IRES-*hRXFP1* construct was inserted in 5' to 3' orientation. (b) To verify homologous integration, we used PCR analysis of the ES cell DNA with one primer designed from the vector backbone and the other designed from exon 15 or 17 outside the targeting construct. DNA from recombinant ES clone (+ lane) produced successful amplification of the 4.2- and 4.1-kb fragments. M, DNA ladder.

B. Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from various tissues using RNeasy Plus kit with an additional RNase-Free DNase step (Qiagen, Valencia, CA), and cDNA was synthesized using Verso cDNA kit (Thermo Scientific, Waltham, MA) according to the manufacturers' protocols. Expression of *Rxfp1* was evaluated by conventional reverse transcription polymerase chain reaction (RT-PCR) and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Go Taq Q-PCR master mix (Promega, Madison, WI) kit was used for real-time qRT-PCR, and primers were designed to be exon-spanning. The primer sequences are as follows: β -actin (*Actb*) forward, CTAAGGCCAACCGTGAAAAG, and reverse, ACCAGAGGCATACAGGGACA; mouse *Rxfp1* (for RT-PCR) forward mRxfp1ex15F-ki and reverse mLgrEx17R (primer sequences are described previously); mouse *Rxfp1* (for QRT-PCR) forward mRxfp1ex17F, CTGTGCTGGATTCCCATCTT, and reverse mRxfp1ex18R, GTTGTGCCAGAGTTGATGGA; human *RXFP1*: hLGR7ex1/2bpF and hLGR7ex2/158bpR (see previously). The RNA isolated from human monocytic cell line THP1 (ATCC, Manassas, VA), which expresses endogenous RXFP1, was used to check the specificity of the human primers. The SybrGreen real-time protocol was run on an Eppendorf Mastercycler ep realplex instrument (Eppendorf, Westbury, NY). Relative fold change in messenger RNA level was calculated by the comparative C_t ($2^{-\Delta\Delta C_t}$) method, where β -actin expression was used for normalization. The number of animals used is indicated in figure legends.

C. Phenotypic Analysis of Pregnant Females

The effects of human *RXFP1* knock-in were studied in late pregnant females at day 18.5 after vaginal plug detection as described previously [8]. Females were euthanized by CO₂ overexposure and their reproductive organs measured, collected, and fixed in 4% paraformaldehyde. The length of the pubic symphysis was determined using a dissecting microscope equipped with an ocular micrometer [14]. Vaginal and pubic ligament sections at 6 to 7 μ m were stained using Masson's Trichrome Stain kit (Sigma-Aldrich Corp., St. Louis, MO) according to the manufacturer's protocol. The thickness of vaginal epithelial cell layers was measured in 10 random cross sections for each sample of three to four females per group using AxioVision Software (Carl Zeiss Microscopy, Jena, Germany) on Carl Zeiss Axio A1 Microscope equipped with an AxioCam MRc5 CCD camera. The number of animals analyzed is indicated in figure legends. Previously published data for the wild-type, heterozygous *+Rxfp1-LacZ*-, and *Rxfp1*-deficient females of the same mutant line as in the current experiments were used for comparisons [8]. Differences were expressed as mean \pm standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison test using GraphPad software (La Jolla, CA).

D. Immunohistochemical Analysis

The testes from adult males and pubic symphyses from pregnant females on day 18.5 after detection of the vaginal plug were collected, fixed, embedded in paraffin, and then sectioned using standard procedures. Immunohistochemistry (IHC) staining was performed on deparaffinized and rehydrated tissue sections using anti-RXFP1 antibody (1:2000, A.9227.1, RRID: AB_2650494; Immundiagnostik AG, Bensheim, Germany) and anti-RXFP1 antibody (1:100, sc-50328, RRID: AB_218472; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described [15]. The IHC staining was revealed by the ImmPACT DAP substrate (Vector Laboratories, Burlingame, CA), producing brown staining. The nuclei were stained with Harris' Alum Hematoxylin (EMD Millipore, Billerica, MA).

E. Stimulation of HR

The effect of RLN and ML290 on HR was measured in unconscious mice. One microgram of human recombinant RLN (PeproTech Inc., Rocky Hill, NJ) was diluted in 65 μ L phosphate-buffered

saline with 8% dimethyl sulfoxide, 8% NMP (Fisher Scientific, Pittsburgh, PA), 8% Kolliphor HS15 (Sigma-Aldrich), and 8% PEG400 (Fisher Scientific) and used for injections (1 μ g RLN/25 g body weight). The same vehicle was used for ML290 (100 μ g/25 g body weight) and control (dimethyl sulfoxide) injections. Adult 4- to 6-month-old females of different genotypes were used in this analysis. Mice were anesthetized with isoflurane via vaporizer in an induction chamber with 5.0% isoflurane in 80% oxygen for 40 to 50 seconds. Anesthesia was maintained via spontaneous breathing of 1.5% isoflurane in 80% oxygen administered via nose cone, and mice were maintained at 30°C to 32°C on a warming pad. HR was measured by tail cuff using a Non-Invasive Blood Pressure System (Kent Scientific Corp., Torrington, CT). Basal measurements were recorded each minute for the first 10 minutes. Treatment was administered via intravenous injection in the tail vein after which HR was recorded for 40 minutes. Changes in HR were calculated by subtraction of average basal HR from HR at every minute for each mouse. These changes were then averaged over each of the following intervals: minute 0, minute 1 to 5, minute 6 to 10, minute 11 to 15, minute 16 to 20, minute 21 to 25, minute 26 to 30, minute 31 to 35, and minute 36 to 40. To compare HR changes within groups at each interval, paired *t* tests were run. To compare HR changes between groups treated with ML290 at each interval, independent *t* tests were run; one-way ANOVAs were run to compare changes in HR between groups treated with RLN. To identify whether the mean difference in HR changed over the nine time points within groups, a repeated-measures ANOVA was run per treatment.

F. Osmolality Assessment

The effect of ML290 on serum osmolality was evaluated in homozygous *hRXFP1/hRXFP1* and wild-type 3-to 5-month old males. Mice were injected intraperitoneally twice at 3-hour intervals with ML290 (1 mg/25 g body weight, n = 6) or with dimethyl sulfoxide (n = 8) in the same vehicle as previously mentioned. Mice were euthanized 7 hours after the first injection. Blood was collected by cardiac puncture and serum samples prepared and stored at -80°C. Serum was submitted for analysis to the Division of Comparative Pathology, Department of Pathology & Laboratory Medicine, University of Miami Miller School of Medicine, Miami, Florida. Glucose (Glu), blood urea nitrogen (BUN), and sodium (Na) concentration were determined on an Ortho Vitros 250 analyzer (Ortho, Rochester, NY). The osmolality was calculated as = $1.86(\text{Na}) + (\text{Glu}/18) + (\text{BUN}/6)$ [16]. Differences were assessed by Student *t* test.

2. Results

A. Insertion of Full *hRXFP1* cDNA Disrupts *mRxfp1* Transcript

An insertional vector [Fig. 1(a)] was used to introduce human *RXFP1* cDNA into exon 17 of the endogenous mouse *Rxfp1* gene. The IRES element provided translation of the inserted human *RXFP1* cDNA. Along with human cDNA, the backbone of the Bluescript vector and floxed neo cassette was introduced into the mouse genomic DNA. The insertion also created a duplication of the genomic fragment used in the targeting vector, including partial duplications of exons 15 and 17 and complete duplication of exon 16. The recombinant ES cells were identified by long-range PCR with primers specific for the vector and the sequence of exons 15 and 17 outside the targeting construct [Fig. 1(b)]. Chimeric males were bred with wild-type females, and germ-line transmission was obtained in several litters. The heterozygous progeny *hRXFP1/+* had normal fertility and viability. To produce mutant homozygotes, we bred *hRXFP1/+* females with males homozygous for an *Rxfp1-LacZ* knockout allele [8]. The F1 diheterozygous *hRXFP1/Rxfp1-LacZ* mice were intercrossed to produce homozygous *hRXFP1/hRXFP1* animals, which were negative for *LacZ* DNA. All animals used in these crosses showed normal fertility, females were able to deliver and nurse their pups, and segregation of the various alleles in all crosses was Mendelian (data not shown).

B. *hRXFP1* Is Expressed Similarly to *mRxfp1* in Tissues of Interest

First, using homozygous *hRXFP1/hRXFP1* males, we showed that insertion of the targeting vector in mouse *Rxfp1* locus disrupted the expression of the endogenous gene. Mouse *Rxfp1* primers designed outside the targeting region were used in RT-PCR with testis cDNA prepared with RNA from wild-type and homozygous mutant males. As shown in Fig. 2(a), the expected *Rxfp1* amplicon was detected in wild-type but not in humanized mice, suggesting that proper splicing of the mouse *Rxfp1* transcript was disrupted in mutants.

The expression profile of *hRXFP1* in homozygotes was analyzed in several male organs by qRT-PCR and compared with that of the mouse *Rxfp1* allele in wild-type animals. Primers designed for amplification of *hRXFP1* transcript were specific for the human gene [Fig. 2(b)]. As shown in Fig. 2(c), the overall expression pattern of the two alleles was similar in wild-type and humanized males and females. Both *hRXFP1* and *Rxfp1* were well expressed at the RNA level in uterus, heart, and brain. The lower range of expression was detected in the ovary, skeletal muscle, lung, liver, and kidney. Surprisingly, the testis showed a dramatic increase in *hRXFP1* allele expression in humanized mice. Because the *hRXFP1* transcript is produced from intronless cDNA, we tested for the presence of genomic DNA in mutant testicular RNA samples used in cDNA synthesis. No PCR amplification was detected when primers from mouse *Rxfp1* introns were used (data not shown), implying the results are not because of genomic DNA contamination. For comparison, the available data on RXFP1 expression in human organs and tissues are presented in Fig. 2(d) [17].

To analyze whether increased *hRXFP1* expression at the RNA level translated into higher expression at the protein level, we performed IHC on the pubic symphysis of pregnant females and testicular tissue with an antibody raised against a peptide specific for the human receptor (Immundiagnostik) [18] and an antibody recognizing both mouse and human receptors (Santa Cruz). The human-specific antibody produced staining in sections of humanized *hRXFP1/hRXFP1* females [Fig. 3(b)] and male tissues [Fig. 3(h)] but not in wild-type [Fig. 3(a) and (g)] or knockout mouse tissues [Fig. 3(c) and (i)]. In the pubic ligament, nonnuclear staining was detected in many cells. In testis, strong staining was detected in Leydig cells and in late spermatids. A similar expression pattern was detected when the second antibody was used [Fig. 3(d–f) and (j–l)]. There was no visual difference in stain intensity between testicular sections from humanized and wild-type mice with the latter antibody, suggesting both alleles are expressed similarly at the protein level.

C. *hRXFP1* Complements the Deletion of *mRxfp1* Allele

Rxfp1-deficient females show several reproductive abnormalities [6, 8]. We analyzed whether substitution with *hRXFP1* complemented the ablation of mouse *Rxfp1*. During parturition in *Rxfp1*-deficient females, the pubic symphysis fails to elongate, resulting in the inability to deliver pups. Pregnant humanized females were able to deliver pups normally, and direct measurements of the pubic ligament at day 18.5 of pregnancy in *hRXFP1/Rxfp1-LacZ* diheterozygotes showed the same length as in *+/Rxfp1-LacZ* and wild-type females (Fig. 4). In *Rxfp1*-deficient females, this distance was significantly shorter ($P < 0.001$) than that of all other groups.

The cervical and vaginal epithelium of *Rxfp1*-deficient pregnant females is much less differentiated than in wild-type females [8]. We have shown that the width of the epithelial layer in vaginal sections was highest in females with both wild-type alleles (Fig. 5). *+/Rxfp1-LacZ* heterozygotes and *hRXFP1/Rxfp1-LacZ* diheterozygotes showed similarly shorter widths than in wild-type females. In *Rxfp1*-deficient females, the width of the vaginal epithelium was significantly shorter than in wild type ($P < 0.001$) and both hetero- and diheterozygotes ($P < 0.01$) (Fig. 5). Masson trichrome staining also revealed the dense collagen deposition characteristic of *Rxfp1*-deficient vaginal epithelium was absent in mice with the human allele (Fig. 5).

Finally, all *hRXFP1/Rxfp1-LacZ* and *hRXFP1/hRXFP1* females fed their pups, and no unusual postnatal lethality was detected in their litters. The size of mammary nipples

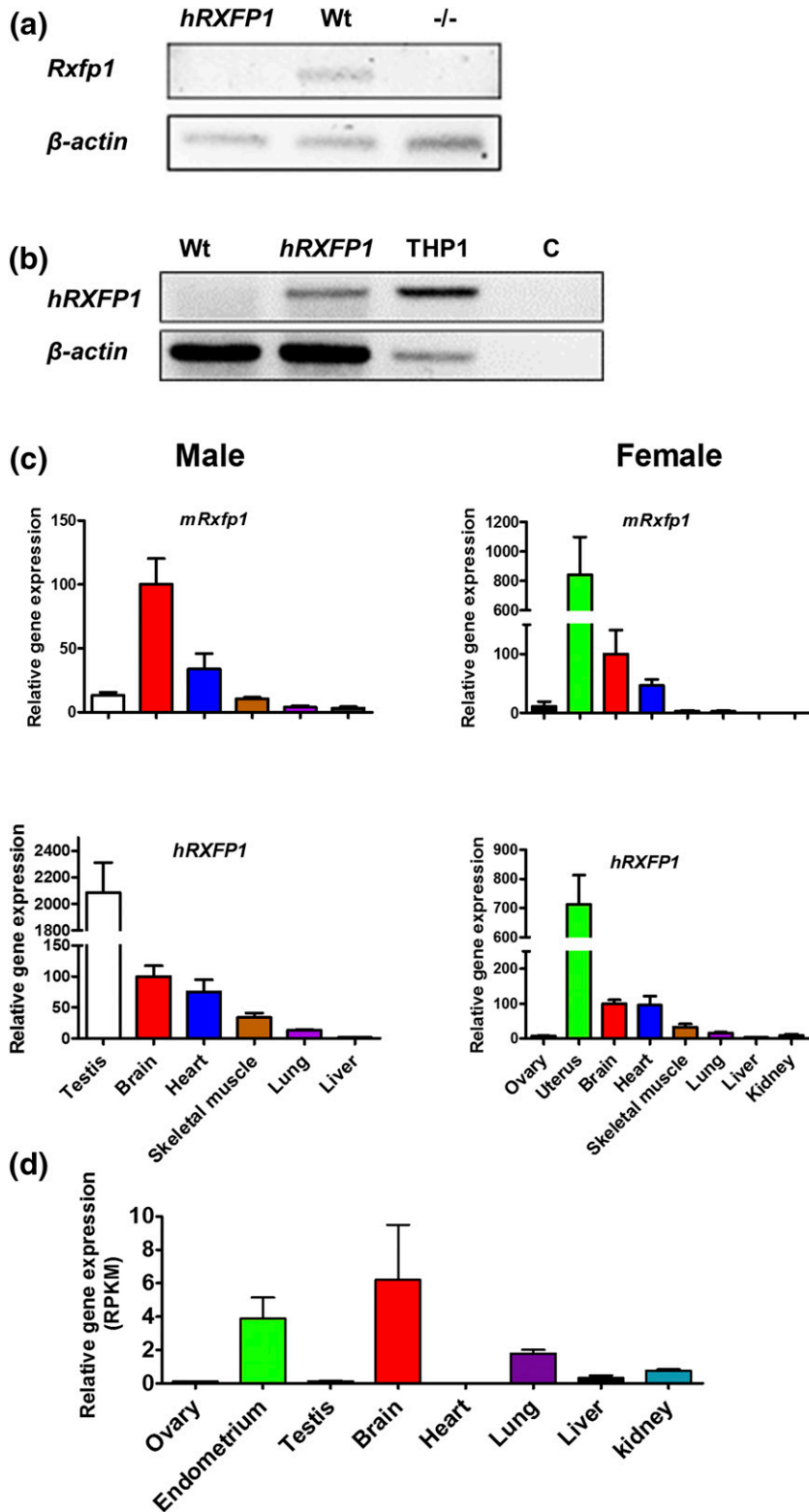


Figure 2. Analysis of *hRXFP1* expression in humanized mice. (a) The expected mouse *Rxfp1* RT-PCR amplicon was detected in RNA isolated from wild-type (Wt) but not in homozygous humanized (*hRXFP1*), or *Rxfp1*-deficient (*-/-*) testes, indicating an ablation of the endogenous gene in humanized mice. (b) The specificity of *hRXFP1* primers was tested with qRT-PCR with brain RNA isolated from wild-type (Wt) and homozygous humanized (*hRXFP1*) mice. Human RNA from THP-1 cells was used as a positive control (THP1). C,

negative (no cDNA) control. (c) qRT-PCR analysis of mouse (*mRxfp1*) and human (*hRXFP1*) gene expression in organs from wild-type (top) and homozygous humanized (bottom) males (left side) and females (right side). Gene expression was normalized to the expression of *Actb* (β -actin) and shown relative to gene expression in the brain. Note high expression of *hRXFP1* in testis RNA. Mean \pm standard error of the mean (n = 4). (d) *RXFP1* RNA-sequence gene expression in human organs and tissues derived from National Center for Biotechnology Information data [18]. RPKM, reads per kilobase per million reads placed. Mean \pm standard error of the mean (n = 2 to 7).

affected in nursing *Rxfp1*^{-/-} females [6] in humanized females was similar to those of wild-type females (data not shown).

D. Injections of RLN and ML290 in Humanized Mice Cause Increase of HR

Intravenous injection of RLN has been shown to increase HR in rats [19]. To compare HR changes in wild-type and humanized *hRXFP1* mice, we treated wild-type, humanized, and *Rxfp1*-deficient females with human RLN. Intravenous injection of RLN in unconscious mice

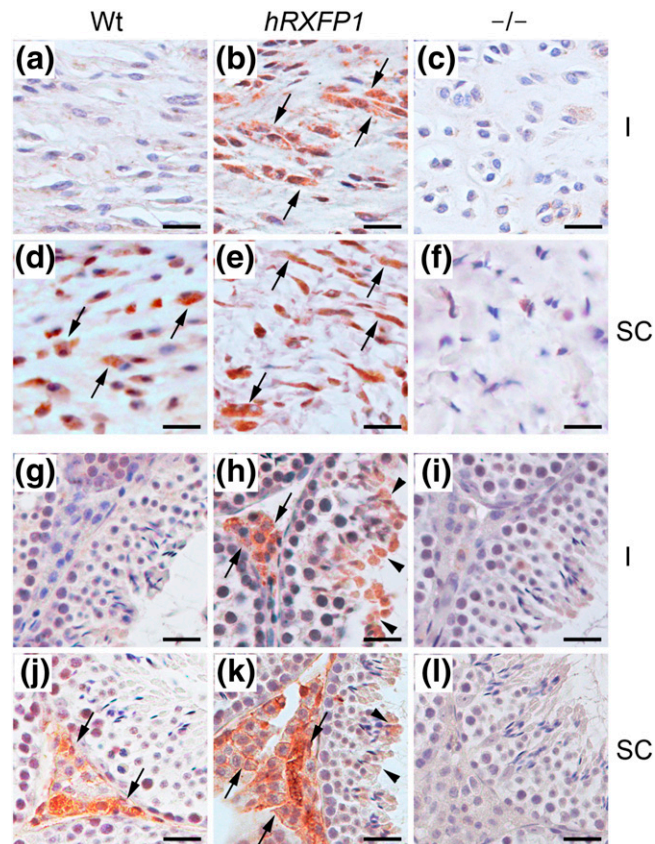


Figure 3. IHC of RXFP1 expression in wild-type (Wt), humanized (*hRXFP1*), and *Rxfp1*-deficient (*-/-*) mice. (a–f) Expression of RXFP1 in pubic ligament (arrows) was revealed with human specific anti-RXFP1 antibody (I) from Immundiagnostik AG (a–c); or nonspecies specific anti-RXFP1 antibody (SC) from Santa Cruz Biotechnology (d–f). Note an absence of brown staining in wild-type and *Rxfp1*-deficient mice with the human-specific antibody. (g–l) Expression of RXFP1 in testis. No staining was detected in wild-type and *Rxfp1*-deficient testes with human-specific antibody. Specific staining was detected in Leydig cells (arrows) and in late spermatids (arrowheads) with human-specific antibody in humanized mice. Representative images of at least two analyzed animals of each genotype are shown. Scale bar, 20 μ m.

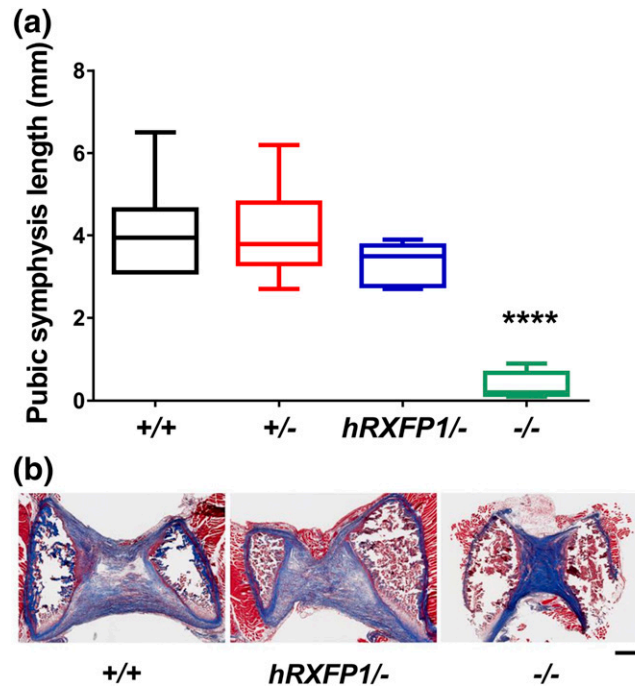


Figure 4. Public ligament length and collagen deposition is similar in pregnant wild-type (+/+), heterozygous (+/-), and diheterozygous humanized mice (*hRXFP1*^{-/-}). (a) Measurement of pubic ligament at day 18.5 of pregnancy (mean \pm standard error of the mean; n = 6 to 15 per group). There was no significant difference between +/+, +/-, and *hRXFP1*^{-/-} mice. The public ligament of *Rxfp1*-deficient (-/-) mice was significantly shorter than the other genotypes (**** $P < 0.001$). (b) Representative images of the pubic ligament stained with Masson trichrome showing collagen in blue. Normal collagen remodeling is observed in +/+ and *hRXFP1*^{-/-} mice, whereas -/- females show characteristically aberrant dense collagen deposition. Scale bar, 500 μ m.

increased HR in wild-type and humanized mice but not *Rxfp1*-deficient mice [Fig. 6(a)]. Paired *t* tests showed that within the wild-type group, the change in HR was significantly different at all intervals after the beginning of treatment ($P < 0.01$). In the humanized group, change in HR was slightly delayed and was significantly different from baseline between 21 and 40 minutes ($P \leq 0.04$). In the knockout group, HR slightly decreased during the 1- to 5-minute interval, returning to basal levels during subsequent measurements. *Post hoc* Tukey tests showed that the wild-type group was significantly different from both the knockout ($P < 0.01$) and humanized ($P < 0.01$) groups at all intervals. From minute 21 to 40, the knockout group was significantly different than both the wild-type group ($P < 0.01$) and the humanized group ($P \leq 0.03$). A repeated-measures ANOVA with Greenhouse-Geisser correction was run to identify the mean difference in HR. The treatment had a significant effect on the change in HR from baseline ($P < 0.01$). There was also a significant interaction between time and genotype ($P < 0.01$) and a significant genotype effect ($P < 0.01$), indicating that the treatment had a different effect on at least one of the three genotype groups. A *post hoc* Tukey test showed that the wild-type group was significantly different from the knockout group ($P < 0.01$) and borderline significantly different from the humanized group ($P = 0.05$).

Next, we analyzed the effect of ML290 on HR. ML290 stimulated human RXFP1 but not mouse RXFP1 *in vitro* [11]. Similarly, ML290 increased HR in humanized but not wild-type mice [Fig. 6(b)]. Paired *t* tests to compare HR changes within groups showed HR in the humanized group was significantly different from minute 0 to the minute 21 to 25 interval ($P = 0.04$), and from minute 26 to 40 ($P \leq 0.02$). However, within the wild-type group, change in HR was not significantly different from HR at minute 0 at any interval. Independent *t* tests to compare HR changes between groups at each interval show that change in HR from their respective baselines between the humanized and wild-type groups were significantly

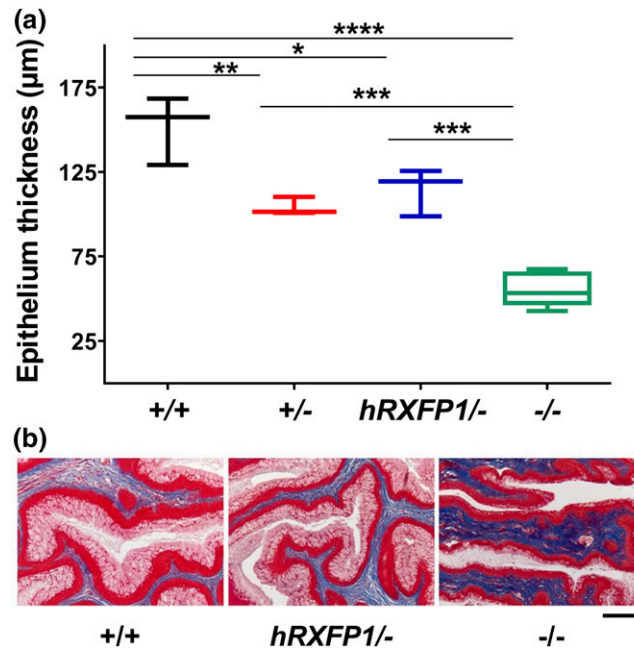


Figure 5. Humanized mice show normal vaginal epithelium differentiation at day 18.5 of pregnancy. (a) Heterozygous mice (+/-) and diheterozygous humanized mice (*hRXFP1*^{-/-}) showed similarly shorter vaginal epithelium width compared with wild-type (+/+) females (** $P < 0.01$ and * $P < 0.05$) (mean \pm standard error of the mean; $n = 3$ to 4 per group). Vaginal epithelium width in *Rxfp1*-deficient females was significantly shorter than in wild-type (**** $P < 0.0001$) and both hetero- and diheterozygotes (*** $P < 0.001$). (b) Representative images of Masson trichrome staining of vaginal epithelium showing collagen in blue. Normal differentiation and collagen remodeling is observed in +/+ and *hRXFP1*^{-/-} mice at day 18.5 of pregnancy. -/- females show characteristically poor differentiation of the epithelium and dense collagen deposition. Scale bar, 200 μm .

different from minute 26 to 40 ($P \leq 0.04$). The treatment had a significant effect on the change in HR from baseline ($P < 0.01$, repeated-measures ANOVA with a Greenhouse-Geisser correction). There was also a significant interaction between time and genotype ($P = 0.04$) and a significant genotype effect ($P = 0.04$), indicating that the treatment had a different effect on the two genotype groups.

E. ML290 Reduces Serum Osmolality in Humanized Mice

It was previously reported that chronically administered RLN decreases serum osmolality in conscious adult, intact female rats [20]. We injected humanized male mice twice with ML290 at 3-hour intervals and collected blood after another 4 hours. Intraperitoneal injections of ML290 resulted in substantial reduction of serum osmolality compared with the vehicle control (Fig. 7).

3. Discussion

Several mouse models of human disease have been used to study and predict possible therapeutic effects of RLN [3]. Native porcine RLN was initially used but subsequently replaced by recombinant human RLN [1]. In most *in vitro* assays, RLN, regardless of species origin, generated the same signaling responses, including cyclic adenosine monophosphate activation in cells transfected with the mouse receptor [21]. It is less clear whether nonmouse RLN can fully engage mouse receptor *in vivo* or how the endogenous mouse RLN will activate transgenic human RXFP1 in live mice. In this study, we produced humanized *RXFP1* mice where we substituted the mouse receptor with the human one. We found that these mice

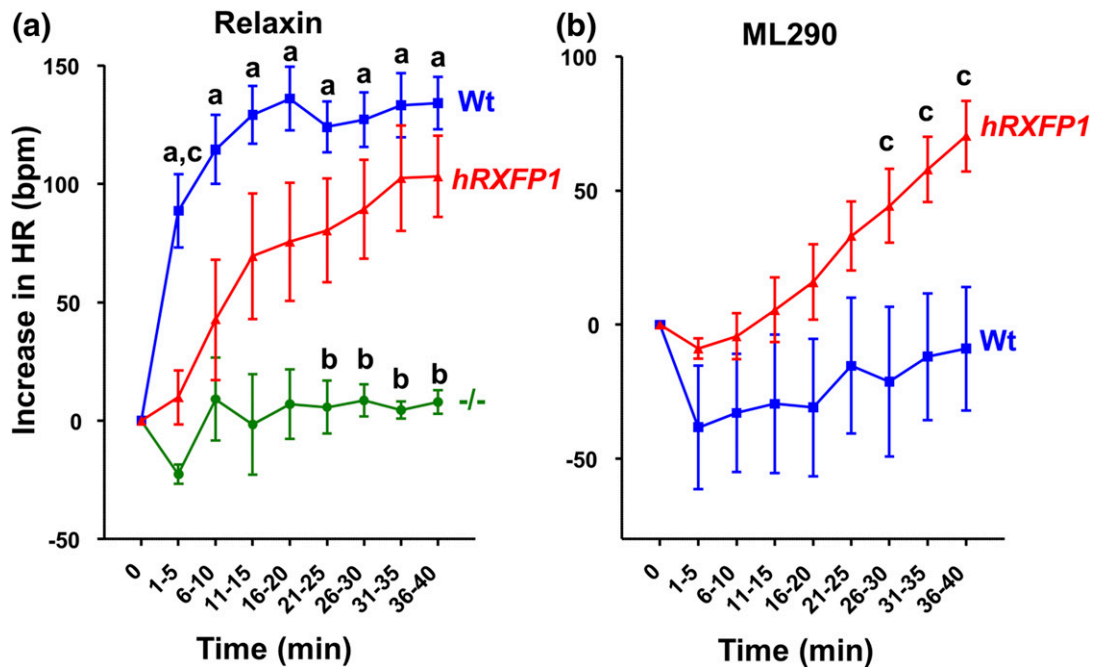


Figure 6. HR increases in response to intravenous (IV) injections of human RLN and ML290 in humanized mice. (a) Effects of IV injection of RLN (1 μ g/25 g body weight) on HR in unconscious wild-type (Wt), homozygous humanized (*hRXFP1*), and *Rxfp1*-deficient (*-/-*) females. Change of HR (beats per minute) after RLN injection over the pretreatment average is shown. HR in Wt mice increases significantly faster than in *hRXFP1* mice (c, $P \leq 0.032$) and *-/-* mice (a, $P \leq 0.013$). RLN also significantly increases HR in *hRXFP1* but not *Rxfp1*-deficient mice (c, $P \leq 0.009$), similarly to Wt mice. (b) IV injection of ML290 (100 μ g/25 g body weight) increased HR in unconscious *hRXFP1* females but not Wt females (c, $P \leq 0.041$). Mean \pm standard error of the mean (n = 4 to 7).

have a normal reproductive phenotype. Our data support the notion that the human receptor complements the absence of the mouse receptor and can be activated by endogenous RLN. Moreover, RLN-like responses were also demonstrated after ML290 injections, suggesting target engagement with the human receptor.

The initial data on the therapeutic effects of RLN in acute heart failure patients revived interest in this hormone [9] as a cardiovascular and antifibrotic drug. On the other hand, RLN has several disadvantages common for many peptide-based therapeutics. The low *in vivo* stability requires constant delivery of RLN through intravenous injections or, in animal experiments, by osmotic pump. The cost of recombinant peptide production is not trivial. Moreover, there were reports that the prolonged use of native porcine and recombinant human peptide in rats and mice induced an immune response which required higher doses of RLN in chronic use experiments [3]. Development of a small molecule RXFP1 agonist would overcome these problems. However, neither ML290 nor other compounds in this series activated cyclic adenosine monophosphate production in cells transfected with mouse *Rxfp1*. Moreover, ML290 partially suppressed RLN-induced activation of mouse *Rxfp1*, behaving as an allosteric inverse agonist [13]. Thus, we produced a mouse mutant with a humanized *RXFP1* allele suitable for therapeutic testing of human-specific small molecule agonists *in vivo*. This model satisfied four main requirements: (1) the endogenous mouse gene was completely inactivated; (2) *hRXFP1* mirrored expression of the endogenous mouse allele; (3) *hRXFP1* complemented ablation of *mRxfp1*; and (4) ML290 selectively produced quantifiable RLN-like response in these mutants which would allow to compare distinct RXFP1 agonists.

Extensive mutagenic analysis of the human and mouse receptor lead us to identify the third extracellular loop and the 7th transmembrane region as critical for ML290 binding and receptor activation [13]. Indeed, the substitution of two amino acids in this region of the mouse

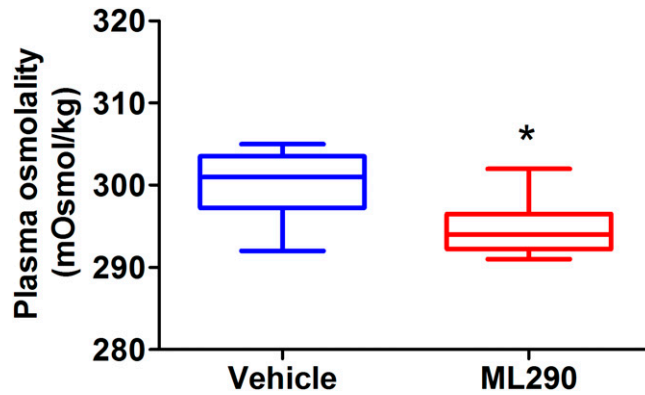


Figure 7. Decrease in serum osmolality after ML290 intraperitoneal (IP) injections in humanized mice. Two IP injections of ML290 (1 mg/25 g body weight, n = 6) or vehicle control (n = 8) were administered at 3-hour intervals. Seven hours after the first injection, the osmolality of humanized mice treated with ML290 was significantly lower than the osmolality of humanized mice treated with vehicle ($P = 0.0257$). Mean \pm standard error of the mean.

receptor with their human receptor counterpart was sufficient to render ML290 effective on the mouse receptor. Because ML290 can activate such a mutated mouse receptor, one possibility was to substitute only exons or part of the exons coding for third extracellular loop with a human sequence. We have, in fact, tried this strategy (data not shown). A construct was made to replace exon 17, the intron, and exon 18 with a single exon combining 17 + 18 sequences, where exon 17 and five base pairs of exon 18 were replaced with the human sequence. Mice with such substitution were produced, and the correct genomic structure was thoroughly verified. However, the resulting mutated allele was not expressed at the RNA level, and the homozygote mutant females were not able to deliver their pups (data not shown). This suggested that either intron 17 contains the regulatory element required for gene transcription, or that the chimeric mouse/human RNA was not stable. An additional drawback was that the production of mice with such a ligand-specific chimeric receptor would have limited use for testing only ML290 or related compounds. We therefore decided to produce mice with the full-length human receptor. Two approaches are generally used to make such animals: an insertion of full-length cDNA into the first exon of the targeted gene; or an insertion of an IRES-cDNA construct into one of the exons to drive translation in a 7-methyl-guanosine cap-independent manner. The latter strategy was used here to account for a potential presence of intron-located regulatory transcriptional elements. In fact, we successfully used an identical design where a LacZ reporter was inserted into *Rxfp1* [6]. These *Rxfp1-LacZ* mice had the phenotype of *Rln1*- or *Rxfp1*-deficient mice [5, 7], indicating the disruption of endogenous gene production, as well as the *Rxfp1* tissue-specific expression of the reporter.

Comparing expression patterns of the human allele in mutant homozygotes with that of the mouse allele in wild-type mice revealed similar patterns in all organs tested except testis. However, the dramatic overexpression of *hRXFP1* in testis was detected at the RNA but not protein level when evaluated by IHC using a species nonspecific RXFP1 antibody. Although we do not have a clear explanation for this phenomenon, one possible reason could be that the presence of the IRES element attracts a specific set of RNA binding elements present in the testis that stabilize messenger RNA, but do not increase its translation. Although no detectable differences in fertility of the mutant males or histologic appearance of testis were detected, further analysis of the effect of the transgene RNA overexpression on the phenotype of humanized males is required.

Using the NCBI publicly available data [17], we compared the *RXFP1* expression in human tissues and organs with mouse gene expression. As in mice, strong *RXFP1* expression in the human brain was detected. In mice, the strongest site of gene expression is the uterus, where it is mostly expressed in the myometrium [8]. This might explain a relative difference in expression in whole mouse uterus *vs* somewhat lower *RXFP1* expression in human

endometrium. Similarly, in our experiments, we used RNA isolated from heart and both atria, whereas it is not clear which part of the human heart was used for analysis. However, the overall expression profile of the human and mouse *RXFP1* gene, and humanized allele in mice, was similar.

The first and well-defined mutant phenotype related to RLN deficiency in female mice is their inability to deliver their pups. The pubic ligament in mutants fails to elongate, the reproductive tract epithelium is poorly developed, and the mammary nipples fail to differentiate [5, 6]. Here we used *hRXFP1*^{-/-} hemizygous females to evaluate the activity of a single human allele. Rescue of the mouse gene deficiency in female reproductive organs by a single copy of the human ortholog suggests that the human receptor is stimulated by endogenous mouse RLN and is thus fully functional.

Our previous data showed that after ML290 injection, compound levels in the heart were three to four times higher than in plasma [11]. We demonstrated here for the first time that the injection of ML290 has similar effect on HR *in vivo* to that of RLN. This response was specific for mice with the human receptor and did not affect wild-type animals, confirming species-specific activity of ML290 demonstrated previously *in vitro*. However, there were some differences between the response to RLN in humanized vs wild-type mice, and between the response to RLN vs ML290 in humanized mice. The HR increase after RLN injection was faster and stronger in wild-type mice. Further analysis of *hRXFP1* expression in target tissues in our model and possible species-specific differences in receptor mediated signal transduction are needed. It is possible that the differences in doses and thus activity of the two agonists was important, or that the physical properties of ML290, such as its solubility, might affect the response dynamic. It is clear however that both the natural ligand and small molecule compound demonstrated overall similar physiological responses *in vivo*. Previously reported RLN-induced changes in blood osmolality [20] were also detected in our model after ML290 injections.

The data presented here demonstrate that the human receptor expressed in mice is functional and responds to both RLN and ML290. Using humanized mice, the antifibrotic, proangiogenic, organ-protective, and other effects demonstrated for RLN can be now tested *in vivo* for ML290 and other agonists for potency and specificity. The *hRXFP1* model is not limited, however, to this series of compounds. Any modulators of the relaxin receptor, including human RLN or its peptide derivatives, can be analyzed for their effect on human RXFP1 in live animals. Previously produced conventional or conditional knockout *Rxfp1* mice [6, 8] should provide further controls to check the specificity of target engagement by various therapeutics.

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