

# GREAT/LGR8 Is the Only Receptor for Insulin-Like 3 Peptide

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During male development testes descend from their embryonic intraabdominal position into the scrotum. Two genes, encoding the insulin-like 3 peptide (INSL3) and the GREAT/LGR8 G protein-coupled receptor, control the differentiation of gubernaculum, the caudal genitoinguinal ligament critical for testicular descent. It was established that the INSL3 peptide activates GREAT/LGR8 receptor *in vitro*. Mutations of *Insl3* or *Great* cause cryptorchidism (undescended testes) in mice. Overexpression of the transgenic *Insl3* causes male-like gubernaculum differentiation, ovarian descent into lower abdominal position, and reduced fertility in females. To address the question whether *Great* deletion complements the mutant female phenotype caused by the *Insl3* overexpres-

sion, we have produced *Insl3* transgenic mice deficient for *Great*. Such females had a wild-type phenotype, demonstrating that *Great* was the only cognate receptor for *Insl3* *in vivo*. We have established that pancreatic HIT cells, transfected with the *INSL3* cDNA, produce functionally active peptide. Analysis of five *INSL3* mutant variants detected in cryptorchid patients showed that P49S substitution renders functionally compromised peptide. Therefore, mutations in *INSL3* might contribute to the etiology of cryptorchidism. We have also showed that synthetic insulin-like peptides (INSL4 and INSL6) were unable to activate LGR7 or GREAT/LGR8. (*Molecular Endocrinology* 17: 2639–2646, 2003)

**I**N MAMMALS, BOTH undifferentiated ovaries and testes are located originally in the abdominal position. During male development, testes descend from this initial position to the scrotum. Two phases of testicular descent have been defined (1). First, the transabdominal phase occurs between 10 and 15 wk of gestation in human embryos and between d 15.5 and d 17.5 post coitum in mouse embryos. An outgrowth of caudal genitoinguinal ligament, gubernaculum, and a regression of cranial suspensory ligament direct movement of the testes toward the inguinal region. Second, the inguinoscrotal phase of testicular descent occurs in humans before birth and in mice within the first 20 d of neonatal development. During this stage, shortening of the gubernacular cord and swelling of the gubernacular bulb provide a passage for the testes into the scrotum. Recently, it was established that two genes encoding the insulin-like 3 peptide (INSL3) and the GREAT (also called LGR8) G protein-coupled receptor (GPCR) control development of gubernaculum. Male mice deficient for *Insl3* (2, 3) or for *Great* (4, 5) exhibit identical high abdominal cryptorchidism. In both cases, gubernaculae fail to differentiate. In males, *Insl3* is produced in pre- and postnatal Leydig cells of testes (6). Expression of the *Great* gene is detected in several organs, with the highest

expression level in gubernaculae, testes, and brain (4, 5). Based on the similarity of the mutant male phenotype, we have suggested that the products of the two genes could, in fact, function as a cognate ligand-receptor pair during development (4, 5).

It was shown that synthetic INSL3 peptide (7) activates GREAT receptor *in vitro*. Closely related to INSL3, relaxin peptide also activates GREAT, as well as another GPCR, LGR7 (8, 9); whereas synthetic INSL3 fails to stimulate LGR7 receptor *in vitro* (7). GREAT and LGR7 receptors exhibit a high degree of homology and belong to the same subfamily of GPCRs as the glycoprotein hormone receptors (4, 5, 8, 9). Expression of two receptors overlaps in several tissues. Both LGR7 and GREAT respond to ligand stimulation through a cAMP-dependent pathway, distinct from that of the structurally related insulin and insulin-like growth factors. The indiscriminate character of relaxin interaction with both LGR7 and GREAT raises the question of specificity of hormone-receptor pairing in the relaxin-like group of peptides. To ascertain the possibility of redundancy of receptors for the INSL3, we designed the study where we examined the role of GREAT in the INSL3 signaling *in vivo*. It was shown earlier that transgenic overexpression of the *Insl3* in females results in outgrowth of gubernaculae and descent of the ovaries into the low intraabdominal position (10). Here we demonstrate that the *Great* deletion rescues the phenotype

Abbreviations: GPCR, G protein-coupled receptor; GREAT, GPCR affecting testis descent; INSL3, insulin-like 3 peptide; LGR, leucine-rich repeat-containing GPCR.

caused by the *Ins13* overexpression, indicating that the Great receptor is the only receptor for *Ins13*.

We have shown previously, that a unique T222P substitution in GREAT detected in a cryptorchid patient renders a nonfunctional protein, unable to respond to the ligand stimulation (5). Mutation analysis of the *INSL3* gene in cryptorchid patients also revealed several unique variants with the single-amino acid substitutions (11–18). Functional significance of these variants remained unclear to date. Our results indicate that mutant INSL3 (P49S) peptide in one of the cryptorchid patients fails to activate the GREAT receptor. Thus, mutation in INSL3 can be causative in the development of the cryptorchid phenotype in humans.

We have also demonstrated that whereas synthetic INSL3 peptide activates GREAT receptor *in vitro*, synthetic INSL4 and INSL6 remain ineffective. Together, these results demonstrate an exclusive role of INSL3 and GREAT in testicular descent.

## RESULTS

### Complementation of the Abnormal Phenotype in *Ins13* Transgenic Mice Deficient for *Great*

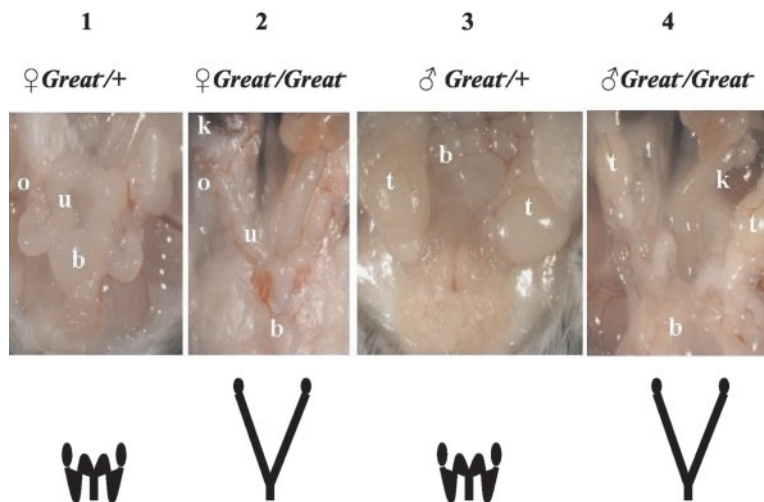
To address the question whether *Great* is the only receptor for *Ins13*, we have produced transgenic mice overexpressing *Ins13* and deficient for *Great*. The phenotypes of the resulting animals are shown in Fig. 1. *Ins13* transgenic females with a functional copy of *Great* (*Tg(Ins2-Ins13) Great-/+*) had the mutant phenotype with ovaries located in the inguinal, low abdominal region. Analysis of the gonadal position in

*Tg(Ins2-Ins13) Great<sup>-/-</sup>Great<sup>-/-</sup>* females revealed that *Ins13* did not stimulate male-like differentiation of gubernaculae in the absence of the *Great* receptor (Fig. 1). Transgenic females overexpressing *Ins13* with the deletion of *Great* had a wild-type phenotype, with ovaries in the normal, high abdominal position; males of the same genotype developed cryptorchidism. Expression of *Lgr7*, related to the *Great* receptor, was clearly detected in the gubernaculum (data not shown). Despite this, *Ins13* could not stimulate gubernacular development in the absence of *Great*.

The other phenotype associated with *Ins13* overexpression is a reduced fertility of the mutant females. It was shown, that in *Tg(Ins2-Ins13)* females there was a loss of embryos due to a fetal mortality during mid-gestation (10). We have compared fertility of the females with or without *Tg(Ins2-Ins13)* transgene (Table 1). *Tg(Ins2-Ins13)* transgenic females with a functional allele of *Great* have reduced fertility as described previously (10). Comparison of the litter sizes of the females deficient for *Great*, with or without *Ins13* transgene, revealed that the two groups have the same fertility (Table 1). Thus, deletion of the *Great* receptor fully complements deleterious effects of the *Ins13* hormone overexpression on female gubernaculae differentiation and fertility.

### Production of Recombinant Human INSL3 in HIT Cells

Previously, it has been shown that ACTH-secreting cells AtT20, transfected with relaxin 3 (*RLN3*) cDNA, produce physiologically active RLN3 peptide (19). In our experiments we used a pancreatic cell line (HIT) to



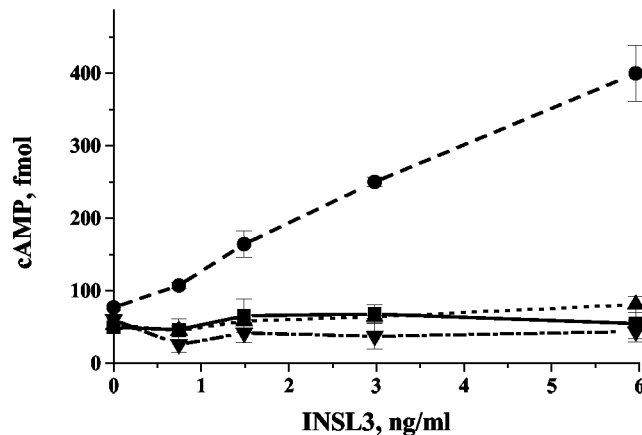
**Fig. 1.** *Ins13* Fails to Stimulate Gubernacular Differentiation in Mice with Deletion of the *Great* Receptor

Phenotypes of the mice with transgenic allele of *Ins13*, *Tg(Ins2-Ins13)*, with or without functional *Great* allele. Overexpression of the *Ins13* transgene causes differentiation of gubernaculae in females and descent of the ovaries to the inguinal region (1). Overexpression of *Ins13* fails to stimulate differentiation of the gubernaculum in females with the deletion of the *Great* receptor; they have a wild-type phenotype (2). *Ins13* transgenic males have a wild-type phenotype (3). Deletion of *Great* in *Ins13* transgenic males causes high intraabdominal cryptorchidism (4). b, Bladder; k, kidney; o, ovary; u, uterus; t, testis. Schematic position of the gonads is shown at the bottom of the figure.

**Table 1.** Deletion of the *Great* Receptor Normalizes Fertility in *Ins13* Transgenic Females

Genotype of the Females	<i>Great</i> <sup>-/+</sup>	<i>Great</i> <sup>-/+</sup> <i>Tg(Ins2-Ins13)</i>	<i>Great</i> <sup>-</sup> / <i>Great</i> <sup>-</sup>	<i>Great</i> <sup>-</sup> / <i>Great</i> <sup>-</sup> <i>Tg(Ins2-Ins13)</i>
No. of litters	9	12	10	11
Litter size	8.4 ± 1.0	6.2 ± 0.6 <sup>a</sup>	8.2 ± 0.5	9.0 ± 0.4

<sup>a</sup> Litter size significantly different between *Great*<sup>-/+</sup> *Tg(Ins2-Ins13)* and other females ( $P < 0.05$ , *t* test).

**Fig. 2.** Recombinant INSL3 Peptide Stimulates GREAT Receptor

Human recombinant INSL3 was produced by transfecting HIT cells with *INSL3* cDNA expression vector. INSL3 hormone content in the conditioned medium was measured with RIA; the medium was used to stimulate 293T cells expressing GREAT (circles) or LGR7 (inverted triangles). The equivalent amounts of medium from HIT cells, transfected with vector DNA, were used as a control (squares and triangles, respectively).

produce recombinant INSL3 peptide and to analyze the ability of this peptide to activate GREAT receptor. HIT cells were transiently transfected with *INSL3* cDNA expression construct. Conditioned culture media from these cells containing secreted hormone were used to treat human embryonic kidney 293T cells, transfected with human *GREAT* or *LGR7* cDNA. It has been established that an activation of the GREAT or LGR7 receptors causes an increase of intracellular cAMP (7, 8). We used such an approach to determine the biological activity of the recombinant peptides. Concentration of the INSL3 peptide in the conditioned medium was determined using INSL3-specific RIA. Figure 2 shows that medium from the HIT cells transfected with a vector DNA did not induce an increase in cAMP concentration in cells expressing GREAT or LGR7. Conversely, medium containing recombinant wild-type INSL3 was able to increase the cAMP level in a dose-dependent manner in cells expressing GREAT. The INSL3-induced activation was specific for the GREAT receptor and did not affect the concentration of cAMP in cells expressing the LGR7 receptor (Fig. 2).

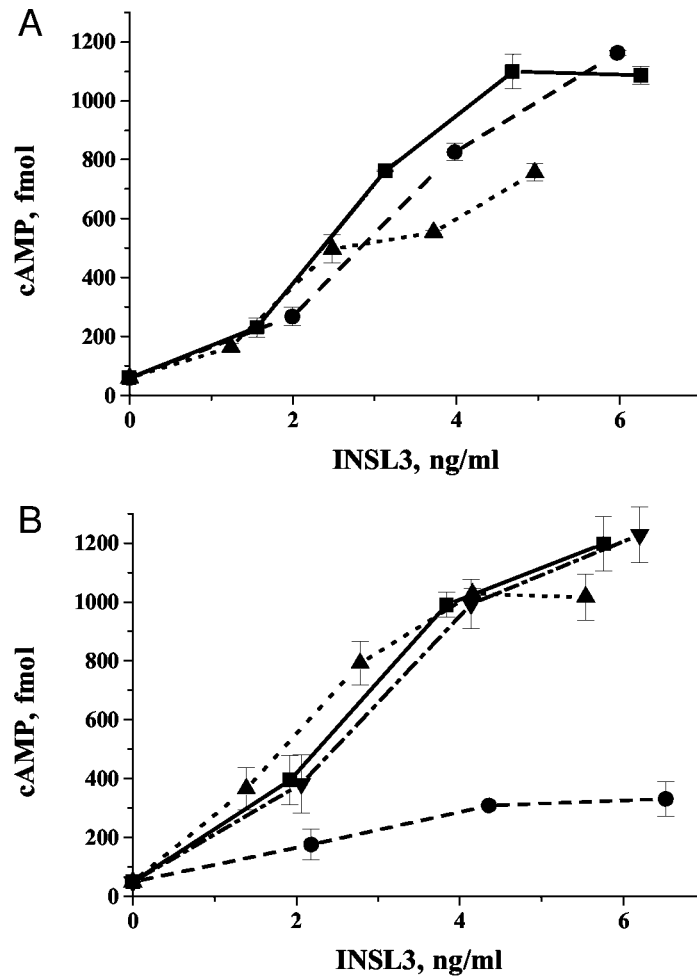
#### Analysis of INSL3 Mutant Variants Associated with Cryptorchidism

Several single-base pair variants of the *INSL3* gene have been found in human populations. To elucidate the physiological relevance of such substitutions, we

analyzed the ability of mutant INSL3 peptides to activate the GREAT receptor. Four mutant variants of *INSL3* (P49S, P93L, R102C, and N110K, where the first amino acid corresponds to the first codon in the full-length *INSL3* cDNA) previously detected in cryptorchid patients (13, 15, 16, 18) and a unique variant (R102H) found in a normal female (16) have been produced using site-specific mutagenesis. Expression constructs of the *INSL3* cDNAs encoding different mutant peptides were introduced into HIT cells. All five constructs produced RNA transcripts of the correct size as evident from the Northern blot analysis (data not shown). The concentration of INSL3 peptides in the conditioned media was determined by RIA, and normalized amounts of media were used to stimulate 293T cells expressing the GREAT receptor. Analysis of cAMP level in INSL3-treated cells revealed that the INSL3 peptide carrying the P49S substitution drastically loses its ability to activate the receptor. R102C substitution only moderately affected INSL3 physiological activity. Neither P93L, R102H, nor N110K substitutions changed the ability of INSL3 to induce cAMP increase in the cells expressing GREAT (Fig. 3).

#### INSL4 and INSL6 Peptides Do Not Activate LGR7 or GREAT Receptors

As was shown before (7, 8), the GREAT receptor can be activated by both INSL3 and relaxin *in vitro*. We



**Fig. 3.** Functional Analysis of Mutant INSL3 Peptides

A, 293T cells, transfected with *GREAT* DNA, were challenged with conditioned media from HIT cells, expressing wild-type INSL3 (squares), P93L mutant (circles), or R102C mutant (triangles). B, 293T cells, transfected with *GREAT* DNA, were challenged with conditioned media from HIT cells, expressing wild-type INSL3 (squares), P49S mutant (circles), R102H mutant (triangles), or N110K mutant (inverted triangles). INSL3 hormone content in HIT media was measured with RIA. Figure shows the results of one of three representative experiments. Each point represents the mean values with a SE of the duplicate measurements.

have studied GREAT activation by two other recently identified relaxin-like peptides, INSL4 and INSL6 (20–22). Receptors and signaling pathways for these insulin-like peptides are unknown. Based on the homology between insulin gene family members, the predicted structure of the A and B chains of INSL4 and INSL6 has been defined (20–22). We used synthetic INSL3, INSL4, and INSL6 peptides with the structure depicted in Fig. 4 to analyze their ability to activate GREAT and LGR7. As shown in Fig. 5, synthetic human INSL3 increased intracellular cAMP level in 293T cells expressing GREAT; however, the activity of such peptide was significantly lower than that of recombinant INSL3 (Fig. 3) or porcine relaxin (data not shown). Synthetic INSL4 and INSL6 were unable to activate GREAT receptor (Fig. 5). None of the used insulin-like peptides was able to activate LGR7 (data not shown). The results demonstrate that synthetic INSL4 and INSL6 peptides do not activate GREAT or LGR7.

## DISCUSSION

Cryptorchidism in humans is one of the most frequent congenital abnormalities. Despite clear indications of the hereditary component in the etiology of the disease, the genetic basis of testicular descent remains vague. Using gene targeting and transgenic approaches, our laboratories recently established that the *Ins3* hormone and G protein-coupled receptor named Great control the first phase of testicular descent in mice (2, 5, 10). It has been shown that transgenic overexpression of *Ins3* in female mice causes male-like differentiation of the gubernaculum and ovarian descent into the inguinal region (10). Transgenic females carrying the *Ins3* gene under the control of the rat insulin 2 promoter (*Ins2-Ins3*) display bilateral inguinal hernia and reduced fertility; transgenic males have a wild-type phenotype. Thus, *Ins3* alone

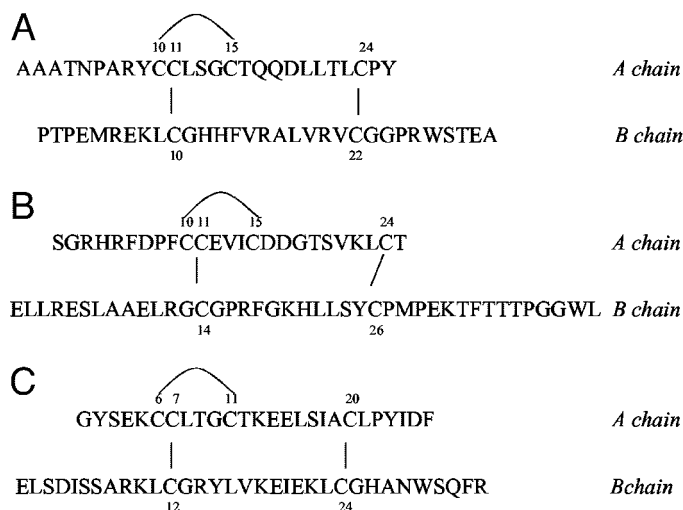


Fig. 4. Primary Structure of Synthetic INSL3 (A), INSL4 (B), and INSL6 (C) Peptides Used in the Experiments

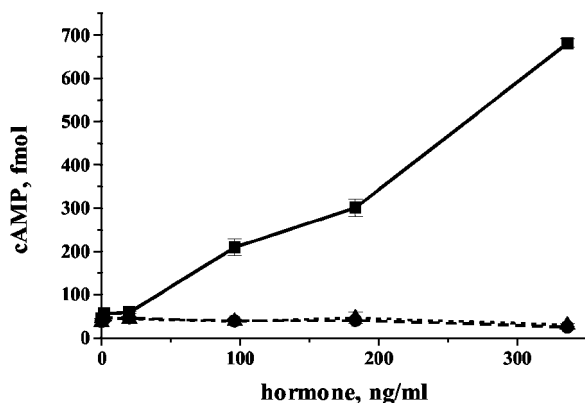


Fig. 5. Stimulation of Cellular cAMP Production by the Synthetic INSL3

Synthetic INSL3 (squares) stimulates dose-dependent cAMP production in 293T cells, transfected with the GREAT cDNA, whereas INSL4 (circles) and INSL6 (triangles) do not. Figure shows the results of one of two representative experiments. Each point represents the mean values with a SE of the duplicate measurements.

controls the first intraabdominal stage of testicular descent in an androgen-independent fashion (10). It was established that both INSL3 and the closely related peptide, relaxin, are capable of activating GREAT receptor *in vitro* (7, 8). To evaluate the role of the Great receptor in the Insl3-mediated signaling *in vivo*, we produced mice that carried the *Insl3* transgene and a null allele of *Great*. If Great is the only receptor for Insl3, overexpression of the transgene in mice with a *Great* deletion will fail to induce gubernacular differentiation in both males and females and, also, restore fertility of the *Tg(Insl2-Insl3) Great*<sup>-/-</sup> females. If Insl3 signals through additional unknown receptor(s), the mutant phenotype caused by transgene overexpression will still be present in *Great*-deficient animals. Analysis of the gonadal position and female fertility in

double-mutant animals clearly showed that the first assumption was correct. Deletion of the *Great* gene completely abrogated the abnormal phenotype associated with the overexpression of Insl3. Therefore, our data prove that the Great receptor is the only receptor responsible for the Insl3-mediated signaling *in vivo*.

Next, we demonstrated that the production of biologically active INSL3 can be achieved in cultured cells transfected with the *INSL3* cDNA. It is generally recognized that members of the insulin superfamily are synthesized as preprohormones; cleavage of signal peptide followed by formation of disulfide bridges and C-peptide exclusion yields the mature hormone. It has been shown previously that the INSL3 peptide produced *in vivo* from the transgenic allele under rat *insulin 2* promoter in  $\beta$ -cells of pancreas is fully functional and complements the genetic deficiency of the endogenous alleles (10). Because the mode of the pro-INSL3 processing is believed to be similar to that of relaxin and insulin (23), we used an insulin-producing pancreatic  $\beta$ -cell line (HIT) to generate recombinant INSL3 and to confirm that such recombinant peptide can stimulate the GREAT receptor *in vitro*. Our results indicate that INSL3 is converted to a mature biologically active form in HIT cells and secreted in the culture medium.

Given the significance of the INSL3-GREAT pathway in testicular descent, the functional analysis of naturally occurring mutations in the *INSL3* gene was the next subject of our study. Mutation analysis of the *INSL3* gene in almost 600 cryptorchid patients has been reported in several published studies (11–18). Some of the described variants of *INSL3* were found both in cryptorchid patients and in a control population of healthy males and, therefore, most likely represent functionally active hormones. Five INSL3 variants (P49S, P93L, R102C, N110K, and R73X) were found only in the cryptorchid patients (13, 15, 16, 18), whereas R102H substitution was found in a single

control female, which, obviously, could not exert the mutant phenotype. Whereas nonsense mutation (R73X) obviously produces a defective peptide, the functional significance of other single-amino acid substitutions was not clear. These five missense mutations can be subdivided into three groups according to the location in pre-proINSL3 structure. The P49S and N110K substitutions are located in the B-chain and A-chain of mature hormone, respectively, whereas P93L and R102C/H are located within the C peptide, excluded from the mature hormone structure. As our data show, only P49S substitution severely reduces INSL3 ability to activate GREAT. A mutated amino acid residue is located in the C terminus of B chain within a highly conserved region of INSL3 (16). All *INSL3* cDNA isolated from different mammalian species contain proline in the position corresponding to P49 in the human INSL3 (24, 25). Interestingly, proline 49 is located within two residues from tryptophan 51, which is critical for the receptor binding (26). Proline 49 is believed to be crucial for the proper orientation of tryptophan 51 because its substitution with D-proline reduces receptor binding 25-fold (26). Thus, the P49S mutation probably affects INSL3 interaction with the receptor.

The P93L substitution, located in the middle of C peptide, leads to a change of less conservative residues (13, 25). Taking into account that horse and rodent INSL3 peptides also carry leucine at the corresponding position, it is not surprising that the P93L mutant effectively activates GREAT. The R102C and R102H substitutions are located at the very end of C peptide in front of the endopeptidase cleavage site. Several mammalian INSL3 peptides contain histidine at the same position. Whereas arginine to histidine substitution does not alter the charge of the region, arginine to cysteine mutation could affect the stretch of positively charged residues necessary for the C peptide cleavage (15). Therefore, decreased efficiency of the R102C variant processing might account for the slight reduction of R102C peptide physiological activity found in our experiments. Despite the fact that N110 is conserved in all known INSL3 peptides, N110K substitution does not change the ability of INSL3 to activate GREAT. In summary, we have shown that at least one substitution (P49S) compromises INSL3 physiological activity, and, therefore, could be responsible for the undescended testes phenotype. Other INSL3 variants do not significantly alter the activation properties of the peptide. It should be noted, however, that other characteristics of the mutant peptides, such as cell-specific efficiency of transcription, translation, processing of the mature protein, or its stability, have not been analyzed in the current study. Nevertheless, taking into account a low frequency of the detected mutations in INSL3, we suggest that alterations in this gene could be responsible for only a small portion of the disease cases in humans.

Considering the promiscuity of the GREAT toward relaxin and INSL3 in the *in vitro* experiments, we an-

alyzed the ability of other related peptides to activate GREAT. It has been reported that recently discovered relaxin 3 peptide (also called INSL7) does not stimulate GREAT receptor but was able to activate relaxin receptor LGR7 (27). Other two members (INSL4 and INSL6) of the relaxin subfamily have been identified recently (20–22). Expression patterns of INSL4 and INSL6 indicate that these peptides, together with INSL3 and relaxin, could be involved in the regulation of reproductive function in mammals. INSL4 (also known as early placenta insulin-like peptide or placentin) is abundant in the placenta (20). INSL6 is found mainly in the testes, specifically within the seminiferous tubules in spermatocytes and round spermatids (22). The endopeptidase cleavage sites in the INSL4 and INSL6 preprohormone sequences were predicted based on the homology with other members of the insulin/relaxin superfamily (20–22). We have demonstrated that neither INSL4 nor INSL6 peptides with the predicted structure activate GREAT receptor in our experiments. However, the inability to check the bioactivity of these peptides due to the lack of data concerning their cognate receptors and signaling cascades may require further experiments to prove this conclusion.

In summary, we have demonstrated that the GREAT receptor is the only cognate receptor for INSL3 *in vivo*. Identification of the specific interaction of the INSL3 with GREAT expands our understanding of the mechanism of testicular descent and cryptorchidism. We have developed an *in vitro* protocol to assess the physiological significance of different INSL3 mutations. Using this method, we have shown that the P49S mutation of INSL3, detected in the cryptorchid patient, renders functionally compromised peptide and therefore can be accountable for the development of the disease phenotype in the affected carrier.

## MATERIALS AND METHODS

### Mouse Breeding and Genotyping

Production and characterization of *Tg(Ins2-Ins13)* transgenic mice (10) and mice deficient for *Great* were described previously (4, 5). *Tg(Ins2-Ins13)* transgenic females were crossed to *Great<sup>ko/+</sup>* heterozygous males, and the resulting *Tg(Ins2-Ins13) Great<sup>ko/+</sup>* were crossed to *crsp/crsp* mice to obtain animals of the *Tg(Ins2-Ins13) Great<sup>ko/crsp</sup>* genotype. *Crsp* mice have a complete deletion of the *Great* gene (4). *Great<sup>ko/+</sup>* heterozygous animals used in this study were the eighth backcross generation of the *Great<sup>ko</sup>* knockout allele onto the FVB inbred strain. Both *Tg(Ins2-Ins13)* transgenic mice and *crsp/crsp* mutants are coisogenic mutants generated and maintained on the FVB inbred background. The genotype of the mice was identified based on the PCR assays with primers specific for *Tg(Ins2-Ins13)* (10), *Great<sup>ko</sup>* (5), and *crsp* (4). Estimations of female fertility have been performed on 2- to 3-month-old females of different genotypes derived from the same litters. The *t* test was used to determine the significance of differences in litter size. The animal studies were approved by Baylor College of Medicine Institutional Committee on animal care.

### Production of the cDNA Expression Constructs

Full-length wild-type *INSL3* cDNA was amplified by RT-PCR from human testis RNA with primers 5'-(CCCAAGCTT)C-CACCATGGACCCCGT-3' and 5'-(CCCAGATCT)GTAGG-GACAGAGGGTCAGCA-3'. The resultant cDNA was subcloned into *HindIII/BamHI* sites of the eukaryotic cell expression vector pcDNA3.1/myc-HisB (Invitrogen, San Diego, CA). Plasmids were purified using the Concert Midi-prep plasmid preparation kit (Life Technologies, Inc., Gaithersburg, MD). The sequence of the construct was verified by sequencing of both strands using gene-specific and vector-derived primers. To produce targeted mutations in the wild-type *INSL3* cDNA we used the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). Resultant cDNA constructs were verified by sequencing of both DNA strands and recloned into pcDNA3.1/myc-HisB vector. *GREAT* cDNA expression vector was obtained previously (5). *LGR7* cDNA was kindly provided by Dr. A. J. W. Hsueh (7).

### Activation of the GREAT and LGR7 Receptors

Porcine relaxin was kindly provided by Dr. O. D. Sherwood, University of Illinois. Synthetic INSL3, INSL4, and INSL6 were obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA). According to the manufacturer, the peptides were synthesized using a previously described method (28); the polypeptide chain synthesis and disulfide bond coupling were verified by mass spectral analysis.

Recombinant wild-type and mutant INSL3 peptides were obtained by transfecting pancreatic HIT cells grown in a T-25 flask with 5  $\mu$ g of the expression construct encoding corresponding peptide using Fugene 6 (Roche, Indianapolis, IN). Medium from cells transfected with pCR3.1 vector was used as a control. The exact concentration of INSL3 peptides in the media was assessed with the INSL3 RIA kit (Phoenix Pharmaceuticals, Inc.) utilizing rabbit polyclonal anti-INSL3 serum raised against full-length synthetic INSL3 peptide.

Activation of the LGR7 and GREAT receptors was assayed as described previously (5). 293T cells grown in 24 wells were transfected with approximately 0.5  $\mu$ g/well of *LGR7* or *GREAT* construct. After 24 h the efficiency of transfection was estimated by the analysis of the secreted AP activity (pAPtag-5 vector from GenHunter, Nashville, TN, was used for cotransfection) with p-nitrophenyl phosphate as a substrate (Sigma Chemical Co., St. Louis, MO). 293T cells were treated with porcine relaxin, synthetic INSLs, or with the HIT conditioned media containing recombinant peptides, in the presence of 250  $\mu$ M isobutylmethylxanthine for 30 min. Cells were harvested, washed, and lysed with cAMP extraction buffer (Amersham Pharmacia Biotech, Arlington Heights, IL). cAMP level was detected using Amersham enzyme immunoassay system (Amersham Pharmacia Biotech). cAMP concentration in each well was measured in duplicate. All experiments were repeated several times using cells from independent transfections.

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