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Generating a sensitive and reliable reporter system for studying INSL3-RXFP2 interactions

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Abstract

Insulin-like peptide 3 (INSL3), a peptide hormone, is primarily expressed in the testis and ovaries. It regulates the inguinal canal translocation of fetal gonad during development through activation of its receptor, RXFP2. Agonists and antagonists of RXFP2 are potential novel medications for contraceptive approaches or infertility treatments. However identification of effective RXFP2 agonists and antagonists was impeded due to the lack of a reliable system to study INSL3-RXFP2 interactions. Here we established such a reporter system named HEK293T-RXFP2 by stable transfection of HEK293T cells with a controllably expressing RXFP2. Compared to transiently transfected cells which were commonly used by previous studies, we found that our HEK293T-RXFP2 cells provided a high sensitive and reliable system to examine the response of RXFP2 to its ligands, as demonstrated by their greater signaling differences of the pEC_{50} and E_{max} values when stimulated by various ligands. Our HEK293T-RXFP2 cells can be used not only to study the functional response of RXFP2, but also to functionally screen the modulators of RXFP2 signaling.

Keywords: Insulin-like peptide 3, RXFP2, cell sorting, cytotoxicity, receptor activation

1. Introduction

Insulin-like peptide 3 (INSL3) is a member of relaxin family peptides comprising two peptide chains cross-linked by three disulfide bonds [1]. It is primarily expressed in the Leydig cells of the testes in males and at lower levels in the thecal cells of the ovaries in females. INSL3 is essential for testicular descent and the control of adult fertility in both male and female through its receptor RXFP2 [2]. Thus, agonists and antagonists of RXFP2 may have considerable potential as specific drugs for novel contraceptive approaches or infertility treatments in both sexes. Recently, INSL3 has been shown to be involved in normal and diseased conditions other than reproduction, ie, bone homeostasis [3], cancer pathogenesis [4] and wound healing [5], indicating that agonists and antagonists of RXFP2 may also be potential medications for these conditions. Thus, identification of specific and effective agonists and antagonists of RXFP2 has significant clinical impact, however such studies are hindered by lack of a reliable reporter system to study the ligand-receptor interaction and its downstream signaling activity.

In most previous studies [6, 7], receptor activation assays were commonly performed by using HEK293T cells with a transient co-transfection of a human RXFP2-expressing construct and a cAMP response element (CRE) -controlled nanoluciferase reporter construct.

However, this method is limited by its low sensitivity and reproducibility, because: 1) the co-transfection of receptor and reporter constructs cannot be guaranteed in all cells; 2) the expression of RXFP2 cannot be controlled, and 3) the receptor overexpression will be toxic to the host cells, or may cause alterations in the downstream signaling pathways. To overcome these problems, we generated a stably RXFP2-transfected HEK293T reporter cell line (HEK293T-RXFP2 cells hereafter) in which the RXFP2 expression can be regulated by doxycycline (Dox) stimulation. The levels of active RXFP2 in such cell line can be controlled by the dosage of Dox used in the culture medium. Our study suggests that our HEK293T-RXFP2 cells can be reliably used to study the functional response of the receptor and serviced as a cellular platform for screening of RXFP2 agonists or antagonists from a peptide library or a compound library.

2. Materials and methods

DNA manipulation

The coding region of enhanced green fluorescent protein (EGFP) gene was PCR-amplified by using pEGFP plasmid as the template, whereas the coding region of RXFP2 gene was PCR-amplified by using pcDNA6/RXFP2 as the template. After confirmation by DNA sequencing, EGFP gene and RXFP2 gene were subcloned into the multiple cloning site 1 (MCS1) and MCS2 sites of a tetracycline inducible expression vector pTRE3G-BI (Clontech, Mountain View, CA, USA), respectively. The resultant construct was designated as pTRE3G-BI/EGFP:RXFP2. In this construct, the expression of both EGFP and RXFP2 genes was controlled by an inducible bi-directional promoter.

pro-INSL3 is a single-chain structure consisting of the B-, C- and A-domains deduced from human INSL3 cDNA sequence. The pro-INSL3 DNA sequence was generated through chemical synthesis (Invitrogen, Shanghai, China). Then the DNA fragment was subcloned into a pET vector. The encoding DNA fragment of the pro-INSL3 was confirmed by DNA sequencing.

Cell culture and transfection

HEK293T cells (ATCC, Manassas, VA, USA) were cultured in complete medium (DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin) at 37°C in a 5% CO_2 incubator. For transfection, the cells were cultured in a 35-mm dish. After reaching ~90% confluency, the cells were co-transfected with the pTRE3G-BI/EGFP:RXFP2 and a pCMV-Tet3G vector (Clontech) by using the Xfect transfection reagent (Clontech) following the instruction provided by the vender. pCMV-Tet3G vector contains a Tet-On 3G gene which express a Dox-response transcription activator.

Purification of HEK293T-RXFP2 cells by FACS

Transfected HEK293T cells were continuously cultured for 8 days, and then were seeded in a 100-mm dish in induction medium (complete medium supplied with 50 ng/ml of Dox) to induce EGFP and RXFP2 expression. After reaching to 95% confluency, the transfected cells were collected and purified by fluorescence-activated cell sorting (FACS) based on EGFP expression. The EGFP positive cells were collected and cultured in the complete medium for ~10 days. To ensure the purity of the transfected cells, we repeated the induction and FACS purification procedure as described above for two more times in order to obtain cells with close to 100% EGFP+. The response of the transfected cells to Dox induction was assessed by fluorescent microscope for EGFP expression as well as ligand binding and activation assays for RXFP2 expression.

Fluorescence microscopic observation

The purified HEK293T cells controllably expressing human RXFP2 (HEK293T-RXFP2 hereafter) were observed by a laser confocal fluorescence microscopy. Briefly, HEK293T-RXFP2

cells were cultured in 35-mm glass-bottomed dishes to ~60% confluence in complete medium. After incubation at 37°C overnight with indicated concentrations of Dox, cells were observed for EGFP expression under a laser confocal fluorescence microscopy with a 488-nm laser excitation.

Preparation of the recombinant wild-type and mutant INSL3s and pro-INSL3

In [B16E]INSL3, [B20E]INSL3, and [B26E]INSL3, three INSL3 mutants, the Arg residues at B16, B20, or B26 sites in the conserved B-chain were replaced by Glu, which influenced a binding affinity towards RXFP2 at different levels [8]. The wild-type and mutant INSL3s were recombinantly prepared as our previous reported [7].

The expression construct of pro-INSL3 was recombinantly expressed in *Escherichia coli* strain BL21 (DE3) star, purified by immobilized metal ion affinity chromatography and in vitro refolded according to our previous procedure [7]. The refolded INSL3 precursor was dissolved in digestion buffer (100 mM Tris/HCl, pH 8.5; 1M guanidine hydrochloride) at a final concentration of ~ 10 mg/mL. Aminopeptidase was then added (emzyme ⁄ peptide mass ratio 1:1000) to remove the additional 6×His tag at the N-terminus and digestion was carried out at 30°C for 16 h. The final reaction mixture was acidified to pH 3.0 using TFA, applied to C18 reverse-phase HPLC, and eluted using an acidic acetonitrile gradient. The eluted peak was manually collected, lyophilized and its molecular mass determined by mass spectrometry.

Circular dichroism (CD) study

The mature wild-type and mutant INSL3s were dissolved in 1.0 mM aqueous HCl (pH 3.0). Their concentrations were determined by UV absorbance at 280 nm using extinction coefficients of ε280 nm, calculated from the number of tryptophan, tyrosine and cystine in the peptides. The final peptide concentrations were adjusted to 0.2 mg/ml for CD measurement that was performed on a Jasco-715 spectropolarimeter at room temperature. The spectra were scanned from 250 nm to 190 nm in a quartz cell with a 1.0 mm path length. The J-700 software for Windows Secondary Structural Estimation (Version 1.10.00) was used for secondary structural content evaluation of the CD spectra.

Receptor binding assays

The receptor binding assays were carried out as we previously reported [8] using HEK293T-RXFP2 cells as the receptor source. Briefly, HEK293T-RXFP2 cells were incubated at 37°C for 48 h in present or absent of 100 ng/ml Dox. The cells were treated with 2 mM of ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 2 minutes, and then washed with serum-free medium. The cell pellets were resuspended in the assay solution (serum-free medium plus 1% BSA) and seeded into 96-well filtration plates (10^5 cells/50 μl/well). After addition of serially diluted INSL3-Luc in 50 μl of the assay solution and incubated at 20°C for 1 h, cells were collected by centrifugation (400 g, 1 min) to remove the assay solution, and washed three times with cold serum-free DMEM medium (200 μl/well). Cell pellets were then resuspended in PBS (100 μl/well) and transferred to a white opaque 96-well plate (50 μl/well). The cells were then lysed in lysis solution (25 μl/well, Promega) for 10 minutes. Finally, 25 μl diluted furimazine substrate was added into each well, and bioluminescence was immediately measured by a SpectraMax M5 plate reader (Molecular Devices) using the luminescence mode. The nonspecific binding was obtained by competition with 1.0 μM of INSL3.

Receptor activation assays

A CRE-controlled nanoluciferase as a reporter [6] was transfected into HEK293T-RXFP2 cells. One day after transfection, the cells were collected and seeded into a 96-well plate in complete medium containing varied concentrations of Dox as indicated. After the cells grew to near 100% confluency (24–48 h), the culture medium was replaced by the activation solution (serum-free DMEM medium plus 1% BSA, 100 μl/well) containing 50 μM 3-isobutyl-1-methylxanthine (IBMX) and indicated concentrations of peptides. The cells were incubated at

37°C for another 5–6 h, the activation solution was removed and the nanoluciferase activity was assayed using the Nano-Glo Luciferase Assay System (Promega) as we have previously reported [7]. The normalized relative nanoluciferase activity data was fitted to a sigmoidal curve using SigmaPlot 10.0 software.

3. Results and discussion

Generation of HEK293T-RXFP2 cells

Overexpression of the receptor RXFP2 in transfected mammalian cells possesses cytotoxicity or possibly changes the downstream signaling pathways, thus we constructed controllably RXFP2-expressing construct using the Tet-On 3G inducible expression system. However, the system requires co-transfection of an exogenous gene cloning plasmid and an expression-controlled plasmid, which leads to low co-transfection efficiencies and large differences between experimental batches. Therefore we intended to establish stably transfected HEK293T cells controllably expressing human RXFP2. In order to facilitate screening of stably transfected cells, we utilized a plasmid that co-expresses both RXFP2 and EGFP reporter genes through a same inducible bidirectional promoter. EGFP can be used as a purification marker for rapid purifying positive cells through flow cytometric sorting. Because of EGFP and RXFP2 gene controlled by a same promoter, the EGFP positive cells should also express RXFP2.

HEK293T cells were co-transfected with pTRE3G-BI/EGFP:RXFP2 plasmid and pCMV-Tet3G plasmid. In most of the transfected cells, exogenous genes couldn't be integrated into the host genome, and would gradually be lost along with cell division; but a small amount of transfected cells (1% or less) could achieve the integration of exogenous genes into the genome, thereby formed stably transfected cells. We enriched the transfected cells by Dox induction followed by FACS for GFP^+ cells. To avoid potential toxicity of long-term RXFP2 overexpression to transfected cells, we started to culture transfected cells without Dox. We only induced the transient expression of RXFP2 and EGFP by Dox at right before the cell sorting. Ten days after HEK293T cells were transfected, ~3000 GFP+ cells can be obtained at the first sorting (the positive rate is ~0.01%, Table 1), indicating that most of the initial transfected cells have lost exogenous plasmids. The cells enriched still expressed exogenous genes. After being amplified in a medium without the inducer, second sorting was conducted. The positive rate was > 0.1%, and increased ~10-fold than the first sorting, indicating that the proportion of stably transfected cells was increased. At the third sorting, the positive rate was > 10%, and increased ~100-fold than the second sorting, indicating that the proportion of stably transfected cells was in a rapid increase, and exogenous genes in host cells gradually became stable by integrating into the host chromosome. Because the expression of EGFP reporter gene in the screened cells can be induced, suggesting that both pTRE3G-BI/EGFP:RXFP2 and pCMV-Tet3G plasmids were stably integrated into the host genome.

Table 1. The summarized results for the flow sorting of transfected cells

receptor	sorting times	total cells	positive cells	positive rate (%)
RXFP2	first	3×10^7	3100	0.01
	second	3×10^7	48300	0.16
	third	10^6	111400	11.14

Verify the controlled expression of EGFP and RXFP2 in HEK293T-RXFP2 cells

After the positive cells obtained at the third sorting were amplified, we firstly observed the expression of EGFP by laser confocal fluorescence microscopy (Fig. 1A). Without the inducer Dox, the expression of EGFP was scarcely observed. Induced with 2.5 ng/mL Dox, a

relatively weak green fluorescence can be observed in most of the cells. Close to 100% of the cells showed strong green fluorescence when induced with 50 ng/mL Dox. This indicated that almost all of these screened cells controllably expressed EGFP reporter gene. Because RXFP2 and EGFP were localized in both sides of a bidirectional promoter, so we speculated that these cells also controllably express RXFP2.

Our recent work provided a novel ultrasensitive bioluminescent INSL3 tracer (INSL3-Luc) for receptor-binding assays [8]. To verify the controllably expression of RXFP2 in our stably transfected HEK293T-RXFP2 cells, we determine the expression of RXFP2 in such cells using INSL3-Luc as a probe. As shown in Fig. 1B, in the absence of Dox, no specific binding of INSL3-luc on HEK293T-RXFP2 cells was detected. Upon 100 ng/ml Dox induction, the specific binding of the bioluminescent INSL3-luc on HEK293T-RXFP2 cells was reliably detected. The binding assays quantitated the expression of functional human RXFP2 on the cell membrane. The measured maximal receptor binding of these stably transfected HEK293T cells ($\sim 1.5\times10^3$/cell) was somewhat less than the value previously measured in the transiently transfected HEK293T cells ($\sim 4\times10^4$/cell) [8]. It can be explained that a host cell may contain multiple copies of plasmids in transiently transfected cells, resulting in high levels of RXFP2 expression. However a stably transfected cell contains only 1-2 copies of plasmids that integrated into the host cell genome, leading to low levels of RXFP2 expression.

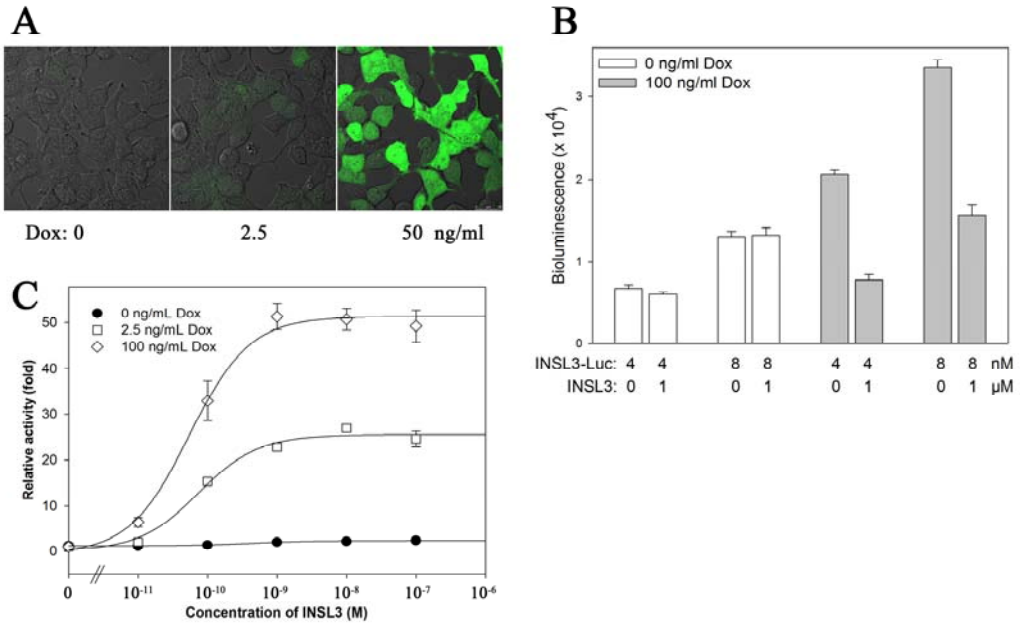

Fig. 1. Dox-induced EGFP expression and RXFP2 activation assays in HEK293T-RXFP2 cells. (A) HEK293T-RXFP2 cells were treated with indicated concentrations of Dox to induce EGFP and RXFP2 expression. EGFP expression was observed by fluorescence microscopic. (B) Receptor binding assays. HEK293T-RXFP2 cells were incubated with 4 nM or 8 nM of INSL3-Luc in the absence or presence of 100 ng/ml Dox for 48 h. 1 μM of unlabeled INSL3 was used as competitor to confirm the specificity of ligand and receptor. Total binding and non-specific binding were shown in bar graph and the data were expressed as mean ± SE (n = 3). (C) Activation of RXFP2 by wild-type INSL3. For the receptor activation assays, HEK293T-RXFP2 cells were induced by varied concentrations of Dox, and a CRE-controlled nanoluciferase was used as reporter. The data were expressed as mean ± SE (n = 3).

The above results indicated that these screened stably transfected cells indeed can induce the expression of RXFP2. But the relatively low levels of RXFP2 expression were not very

suitable for receptor binding assays. Receptor activation assays generally require a few receptors, because overexpression of receptors may be harmful and cause excessive background. Therefore we attempted to implement RXFP2 activation assays using these stably transfected cells. As shown in Fig. 1C, after HEK293T-RXFP2 cells were transiently transfected with CRE-controlled nanoluciferase reporter construct, without Dox induction, INSL3-induced RXFP2 activation could not be detected as demonstrated by the failure in induction of luciferase gene expression. However, upon Dox induction, RXFP2 could be reliably activated in our stably transfected cells by INSL3 as demonstrated by increased expression of luciferase gene. With 2.5 ng/mL Dox induction, the maximum effect (E_{max}) of RXFP2 is ~25-fold of the background (Table 2). While with 100 ng/mL Dox, the E_{max} of RXFP2 is ~50-fold of the background (Table 2). However, the E_{max} of RXFP2 in the transiently transfected HEK293T cells is ~4-fold of the background (Table 2). This study suggested that for receptor activation assays, our stably transfected RXFP2 reporter cells were superior to transiently transfected RXFP2 reporter cells. Due to the comparable pEC_{50} of INSL3-RXFP2 between transiently transfected cells and stably transfected cells (Table 2), the receptor activation curves of INSL3 treatment showed a typical sigmoidal curve in the stably transfected cells (Fig. 1C), suggesting that these stably transfected cells were better and sensitive system for RXFP2 activation assays than the transiently transfected cells.

The stably transfected HEK293T-RXFP2 cells grew well, with normal doubling time and normal morphology, suggesting that controllable expression of EGFP and human RXFP2 were not detrimental to the cell survival and proliferation. After ~10 passages, almost all cells maintained the controllable expression of EGFP, further suggesting that the transfection were stable.

CD analysis

In a recent work [8], using competition receptor-binding assays, we monitored the receptor RXFP2 binding of three INSL3 mutants in which the highly conserved B-chain arginine residues (B16R, B20R, or B26R) were replaced by negatively-charged Glu residue. The receptor binding affinity of [B16E]INSL3 was drastically decreased, while such binding affinities in [B20E]INSL3 and [B26E]INSL3 retained at a relatively high level, suggesting that B16Arg was critical for the receptor binding of INSL3. To test whether HEK293T-RXFP2 cells could discriminate effects of the receptor activation of these mutant ligands, we recombinantly prepared the three INSL3 mutants and the structural changes of these analogs were assessed by CD (Fig. 2). No spectra difference was detected between mutant INSL3s and wild-type INSL3. The a-helix contents of the mutant INSL3s estimated by CD spectra were also comparable to that of wild-type INSL3, suggesting that the mutations in these mutant analogs did not affect their overall structure.

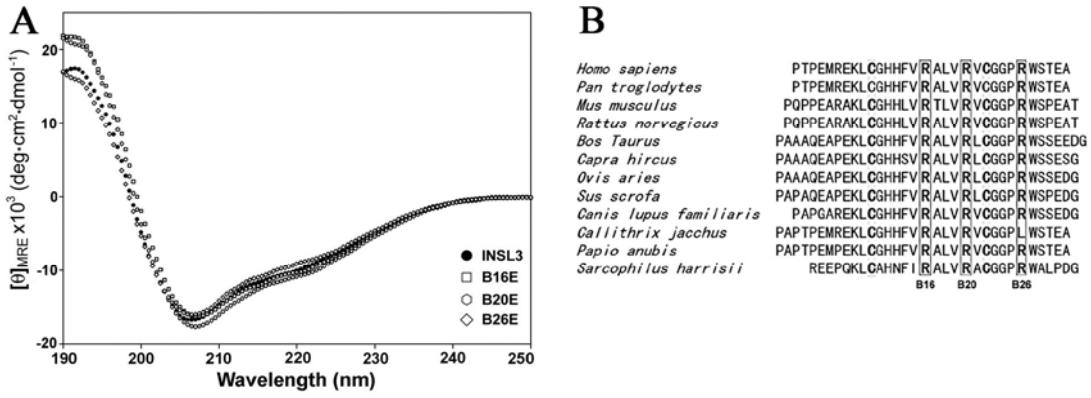

Fig. 2. (A) CD analysis of the mature wild-type and mutant INSL3s. Samples were dissolved in aqueous HCl solution (pH 3.0). (B) The amino acid sequence alignment of the B-chain of INSL3s from different species.

The stably transfected HEK293T-RXFP2 cells were used for receptor activation assays of the mutant ligands

To evaluate the ligand-stimulated response of stably transfected receptor in HEK293T-RXFP2 cells, we measured the levels of the receptor activity upon the stimulation of various ligands (Fig. 3A, B, the calculated pEC_{50} values were summarized in Table 2). After HEK293T-RXFP2 cells were transiently transfected with CRE-controlled nanoluciferase reporter construct and treated with ligands, the receptor activation curves of various ligands showed typical sigmoidal curves in the presence of 2.5 ng/ml Dox (Fig. 3A). However the pEC_{50} and E_{max} values were significantly difference among these mutant ligands (Table 2). [B20E]INSL3 retained a relatively high level of receptor activation efficiency comparable to wild type INSL3. The activation efficiency of [B16E]INSL3 was decreased approximately 100 fold, confirming the critical role of B16Arg in signaling activation. The activation efficiency of [B26E]INSL3 showed a ~40% reduction, suggesting that B26Arg is also involved in stimulating RXFP2 activation. Thus, we speculated that among these three conserved Arg residues, B16Arg and B26Arg are critical for INSL3 interacts with RXFP2. The receptor activation curves of various ligands were also showed typically sigmoidal with 100 ng/ml Dox induction (Fig. 3B), their pEC_{50} values were also similar to that with 2.5 ng/ml Dox. As shown in Fig. 3C, although we were also able to get the same conclusion using transiently RXFP2-transfected HEK293T cells as a receptor source, the pEC_{50} and E_{max} values possessed greater difference in HEK293T-RXFP2 cells in response to wild-type and mutant INSL3s stimulation compared to the transiently transfected cells. We concluded that HEK293T-RXFP2 cells were ~10 fold more sensitive and reliable than the transiently transfected cells (Table 2).

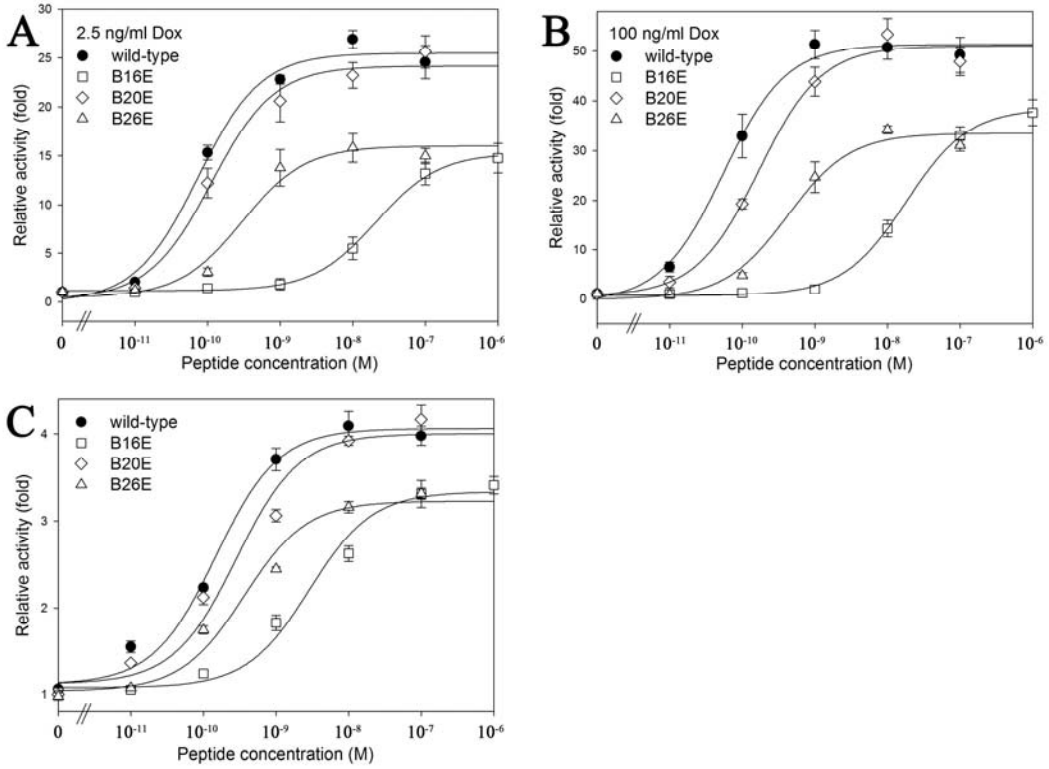

Fig. 3. Activation of RXFP2 by wild-type and mutant INSL3s. Receptor activation assays were induced by 2.5 ng/ml (A), and 100 ng/ml Dox (B) in HEK293T-RXFP2 cells. (C) For the receptor activation assays, the HEK293T cells transiently expressing RXFP2 were used. A CRE-controlled nanoluciferase was used as reporter. The data were expressed as mean ± SE (n = 3) and calculated with sigmoidal curves using SigmaPlot 10.0 software.

The mature form of INSL3 generally was presumed to conform to a two-chain A-B structure. The two-chain INSL3 prepared through chemical synthesis or recombinant expression indeed has very high biological activity [9, 10]. Additionally, natural INSL3 isolated from bovine testis was consisted of A- and B-chain [11]. However, single-chain INSL3s (pro-INSL3) comprising three domains B-C-A were recently isolated from boar and goat testicles with full biological activity [12, 13]. The single-chain INSL3 precursors we recombinantly expressed (the B-chain and A-chain were linked by an eight-residue linker sequence) also retained 40% activity [9]. In order to verify whether pro-INSL3 has very high activity, we prepared human pro-INSL3 by *Escherichia coli* recombinant expression and measured its activation potency toward the receptor RXFP2. As shown in Fig 4A, the activation potency of pro-INSL3 toward RXFP2 was three-fold less than wild-type INSL3 in HEK293T-RXFP2 cells induced by low or high inducer. However, pro-INSL3 still had high activation potency with EC50 at about 0.25 nM (Table 2). Similar results were obtained from transiently RXFP2-transfected HEK293T cells (Fig 4B). Therefore, we concluded that pro-INSL3 had high biological activity but slightly lower than that of two-chain wild-type INSL3.

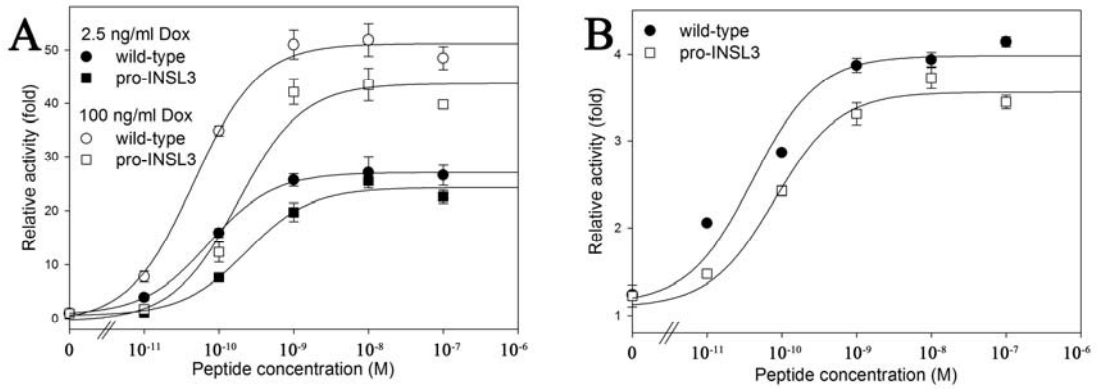

Fig. 4. Activation of RXFP2 by wild-type INSL3 and pro-INSL3. (A) Receptor activation assays were induced by 2.5 ng/ml and 100 ng/ml Dox in HEK293T-RXFP2 cells. (B) For the receptor activation assays, the HEK293T cells transiently expressing RXFP2 were used. A CRE-controlled nanoluciferase was used as reporter. The data were expressed as mean ± SE (n = 3) and calculated with sigmoidal curves using SigmaPlot 10.0 software.

Table 2. The measured pEC_{50} and E_{max} values of wild-type and mutant INSL3s and pro-INSL3

INSL3			wild-type	B16E	B20E	B26E	pro-INSL3
Stable RXFP2	2.5 ng/mL Dox	pEC_{50}	10.14 ± 0.08	7.68 ± 0.14	9.95 ± 0.12	9.53 ± 0.16	9.63 ± 0.11
		E_{max}[a]（fold）	24.6	14.8	25.6	15.0	23.9
	100 ng/mL Dox	pEC_{50}	10.26 ± 0.11	7.75 ± 0.08	9.78 ± 0.10	9.38 ± 0.10	9.67 ± 0.12
		E_{max}[a]（fold）	49.2	37.6	47.9	31.0	43.7
Transient RXFP2	pEC_{50}		9.83 ± 0.07	8.54 ± 0.09	9.55 ± 0.10	9.44 ± 0.08	9.54 ± 0.07
	E_{max}[a]（fold）		4.0	3.4	4.1	3.3	3.5

[a] the maximum magnification of increased reporter gene activities compared to untreated cells.

The controlled assays can be used to study other receptors for screening agonists and antagonists

Cell-based *in vitro* assays, including target gene expression and cell proliferation assays, have been extensively used for the identification and screening of agonists and antagonists for receptors [14, 15]. Although the results obtained from such assays are valuable by using transiently transfected cells, the variation of the results from different experiments might be very big due to the different transfection efficiency. In this study, we generated stably

transfected HEK293T cells with controllable RXFP2 expression. Such reporter cells could sensitively discriminate the activity of various ligands depended their stimulated receptor activation efficiencies. We speculated that our controlled expression method can also be used to generate system for screening agonists or antagonists of other receptors, especially the receptors which are cytotoxic or their activities are dependent on appropriate expression levels. What's more, the cells have advantage of highly sensitivity, which may aid in basic investigations elucidating functions of receptors.

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