

A highly efficient and sensitive screening method for trans-activation activity of estrogen receptors

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Abstract

We describe a highly efficient and sensitive yeast-based screening method for isolating human estrogen receptor α (ER α) mutants with altered trans-activation activity. This method takes advantage of the fact that estrogen receptor is a ligand-activated transcription factor, and links the transactivation activity of estrogen receptor to the growth rate of yeast cells. We used this method to screen a library of human ER α mutants created by random mutagenesis of the ligand binding domain of human ER α in the presence of ligand 17 β -estradiol (E₂). We isolated several human ER α mutants with significantly altered trans-activation activity toward E₂ in yeast cells. We also used this method to screen a library of chemical compounds and showed that it can be used to rapidly identify estrogenic compounds and the different cell growth rates for these estrogenic compounds correlated well with their relative binding affinities. Thus, this method is suitable for selecting novel estrogenic compounds and estrogen receptor mutants. In principle, this method might also be used to isolate mutants of any nuclear receptors with altered trans-activation activity, which may greatly facilitate their structural and functional studies.

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1. Introduction

The human estrogen receptor (ER) (subtypes α and β) is a ligand-regulated transcription factor that mediates the actions of estrogen in different target tissues including the reproductive, pituitary, hypothalamus, bone, liver and cardiovascular system and belongs to the steroid family of nuclear receptors (Katzenellenbogen, 1996). The beneficial effects of estrogens in bone maintenance, on blood lipid profile, and in the cardiovascular system have led to the development of various estrogen pharmaceuticals. In particular, about 40% of breast cancers are estrogen-dependant (Katzenellenbogen et al., 1993), which is the

basis for the therapeutic uses of tamoxifen and other antiestrogens. Unfortunately, despite decades of effort, our understanding of the structure and function of human estrogen receptor is still limited, as evidenced by our inability to rationally design estrogen or antiestrogen pharmaceuticals.

The human estrogen receptor contains a highly conserved DNA-binding domain and a highly conserved ligand-binding domain (LBD). In addition to its ligand binding activity, the LBD also has dimerization activity and a ligand-dependent activation function (AF-2) (Katzenellenbogen, 1996). As described in a simple model, in the absence of ligand, the ER is generally believed to reside in the nucleus of target cells where it is associated with heat-shock-proteins (Hsps) and remains transcriptionally inactive (Smith and Toft, 1993). In the presence of ligand, the ER dissociates with Hsps, forms a stable homodimer and binds an estrogen response element (ERE). This ERE-bound, ligand-occupied ER complex can either activate or suppress transcription of a downstream target gene in a cell- and promoter-specific manner (Fujimoto and Katzenellenbogen,

Abbreviations: ER, estrogen receptor; ER α , estrogen receptor α ; E₂, 17 β -estradiol; RBA, relative binding affinity; LBD, ligand-binding domain; AF, activation function; Hsp, heat-shock-protein; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; PEG, polyethylene glycol; UAS, upstream activating sequence; 3-AT, 3-amino-1,2,4-triazole; DHFR, dihydrofolate reductase.

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1994; Tsai and O'Malley, 1994). In the case that the complex recruits co-activators and induces the transcription of a downstream target gene, this biological function is generalized as the trans-activation or transcriptional activity of ER. However, it should be noted that in the absence of ligand, the ER could also form a homodimer and bind to the ERE (Zhuang et al., 1995), and the ER has been reported to possess substantial ligand-independent ability to activate transcription in vivo (Tzukerman et al., 1990).

Three yeast-based genetic screening methods for isolating ER mutants with altered trans-activation activity have been reported. One method entails the co-transformation of a yeast reporter plasmid containing two or three copies of ERE upstream of the reporter gene *lacZ* and an estrogen receptor expression plasmid (Wrenn and Katzenellenbogen, 1993; Whelan and Miller, 1996; Eng et al., 1997). A library of ER mutants is grown on a selective agar plate containing a specific ligand. The transactivation activity of ER is indicated by the β -galactosidase activity assayed using a chromogenic substrate, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). We had attempted to use this method to isolate human ER α mutants with altered transactivation activity and found that the β -galactosidase activity assay was not very sensitive, time-consuming and low-throughput since it requires multiple steps such as plate replicating, cell lysis and color development. The second method entails the fusion of the estrogen receptor to the GAL4 DNA binding domain which can interact with the GAL4 upstream activating sequence (UAS) upstream of an integrated *GAL1-lacZ* gene (Bush et al., 1996). The addition of a ligand will induce the expression of the *lacZ* gene and can be assayed using X-gal. This method is also not very sensitive and nor amenable for high throughput screening. The third method entails the fusion of the LBD of human ER α to the murine dihydrofolate reductase (DHFR) and the use of a yeast strain lacking a temperature-sensitive essential yeast metabolic enzyme, DHFR (Tucker and Fields, 2001). The binding of ligand to the LBD increases the stability and/or activity of the murine DHFR that leads to increased cell growth. Although this method is sensitive and high throughput, it seems to mainly screen for increased ligand affinity since, for example, estrogenic compound genistein is not active in this assay while it is in the screening method we have developed.

Here, we describe a simple and highly sensitive genetic screen amenable to high throughput isolation of human ER α mutants with altered transactivation activity. This approach is based on the linkage of the transactivation activity of ER to the yeast cell growth. Using this approach, we rapidly identified several novel amino acid substitutions responsible for enhanced transactivation activity of human ER α to 17 β -estradiol (E₂) in yeast cells. We also demonstrated that this method could be used to screen for novel estrogenic compounds.

2. Materials and methods

2.1. Reagents, vectors and yeast strains

Yeast strain YM4271 (*MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *leu2-3, 112*, *trp1-901*, *tyr1-501*, *gal4- Δ 512*, *gal80- Δ 538*, *ade5::hisG*) and integrative reporter vector pHISi-1 were obtained from BD Biosciences Clontech (Palo Alto, CA). pHISi-1 contains the yeast *HIS3* gene downstream of the multiple cloning site and the minimal promoter of the *HIS3* locus (P_{minHIS}). Yeast estrogen receptor expression vector YEpER α containing the wild type human ER α , and estrogen-responsive reporter vector 3ERE-pS2-CAT containing three tandem EREs were kindly provided by Benita S. Katzenellenbogen (University of Illinois, Urbana, IL) (Wrenn and Katzenellenbogen, 1993). All the estrogenic compounds and other chemicals were obtained from Sigma (St. Louis, MO). All the restriction enzymes were obtained from New England BioLabs (Beverly, MA).

2.2. Yeast transformation

Yeast cells were transformed using the lithium acetate/single stranded carrier DNA/polyethylene glycol (PEG) protocol (Agatep et al., 1998). Briefly, overnight yeast culture was inoculated into 50 ml of pre-warmed YPAD medium with a starting cell titer of about 5×10^6 cells/ml and grown to 2×10^7 cells/ml at 30°C on a shaker at 200 rpm (3–5 h). Cells were harvested by centrifugation and washed once in sterile water. The cell pellet was re-suspended in 1 ml of 100 mM LiAc, transferred into a clean 1.5 ml microfuge tube and centrifuged at top speed for 15 s. The cell pellet was re-suspended again with 100 mM LiAc to a final concentration of 2×10^9 cells/ml by vortexing and 50 μ l of cell suspension was then added to a clean 1.5 ml microfuge tube and pelleted by centrifugation. The LiAc solution was carefully removed with a micropipette. A total 240 μ l of PEG MW 3350 (50% w/v), 36 μ l of 1.0 M LiAc, 50 μ l of single stranded salmon sperm carrier DNA (2.0 mg/ml), 0.1–1 μ g of plasmid DNA, and an appropriate amount of sterile water were added to the microfuge tube to make up a final volume of 360 μ l. The sample was mixed by vigorous vortexing and then incubated at 30°C for 30 min followed by heat shock at 42°C for 20 min. Cells were spun down in a microfuge tube and resuspended in 1 ml of sterile water. A proportion of the sample was then plated onto the appropriate selective medium.

2.3. Construction of plasmids and a reporter strain

The reporter plasmid 3ERE-pHISi-1 was constructed by insertion of three tandem copies of estrogen response element (ERE) upstream of the integrated minimal promoter $P_{\text{minHIS}}-HIS3$ reporter gene in plasmid pHISi-1. pHISi-1 contains a bacterial Col E1 origin (*ori*) and the ampicillin

resistant gene for propagation and selection in *Escherichia coli*. The three EREs were prepared from plasmid 3ERE-pS2-CAT using a conventional PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Primers ERE-LP (5'-CGG AAT TCG GTA CCC GGG GAT CTC TTT-3') and ERE-RP (5'-TCC CCC GGG TTC CCA GTC AGG GAT GAG AG-3') were used. The PCR product was purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA), followed by restriction digestion by *Sma*I. The digestion product was purified again, sub-cloned into pHISi-1 vector, and transformed into *E. coli* DH5 α competent cells by heat shock. The reporter plasmid 3ERE-pHISi-1 containing the three EREs without PCR-induced mutations was confirmed by restriction analysis and DNA sequencing of the insert.

The reporter strain was constructed by integrating the reporter plasmid 3ERE-pHISi-1 into the genome of yeast strain YM4271. Plasmid 3ERE-pHISi-1 was linearized in the 3' untranslated region immediately following the *HIS3* marker by restriction enzyme *Xho*I and transformed into yeast strain YM4271. The linearized plasmid was integrated into the non-functional *his3* locus of the YM4271 host strain by in vivo homologous recombination. The reporter strain YM4271HIS was selected on agar plates containing minimal synthetic dropout medium lacking histidine.

2.4. Mutagenesis of human ER α ligand binding domain

Error-prone PCR mutagenesis of the ligand-binding domain of human ER α was performed under the conditions similar to those described (Zhao et al., 1999). Primers ERLBD5N (5'-GAA CAG CCT GGC CTT GTC-3') and ERLBD3C (5'-GTC TCC TCC ACG GAT GCC-3') were used to amplify a 780 bp fragment encoding residues 304–564 (the LBD starts at residue 303 and ends at residue 553). The error-prone PCR reaction contained (100 μ l final volume): 10 mM Tris (pH 8.3 at 25°C), 50 mM KCl, 7 mM MgCl₂, 0.01% (wt/vol) gelatin, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 0.15 mM MnCl₂, 0.3 μ M of both primers, 5 ng of template DNA (plasmid YEpER α), and 5 U *Taq* DNA polymerase (Promega, Madison, WI). PCR was performed in a MJ Research (Waltham, MA) PTC-200 thermocycler for 14 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. The PCR products were purified using QIAEX II gel purification kit (QIAGEN, Valencia, CA) and quantified using gel electrophoresis with EZ Load Precision Molecular Mass Ruler (Bio-Rad, Hercules, CA).

2.5. Construction of a library of human ER α mutants

A library of human ER α mutants was constructed based on the in vivo recombination mechanism of yeast cells (Muhlrad et al., 1992). Briefly, an 84 bp fragment was removed from the human ER α expression plasmid YEpER α by restriction digestion with *Nco*I and *Bgl*II, followed by gel purification of the vector backbone. This cut plasmid

(200 ng) was co-transformed with the mutagenized LBD fragments generated by error-prone PCR (400 ng) into the YM4271 host strain. Because both ends of the mutagenized LBD fragments contained regions homologous to the gapped plasmid, the mutagenized LBD fragments were ligated to the vector backbone as a result of the yeast homologous recombination mechanism. A library of about 10⁴ human ER α mutants was obtained and screened.

2.6. Library screening

Yeast YM4271HIS cells co-transformed with gapped plasmid YEpER α and mutagenized LBD fragments were plated on agar plates containing minimal medium lacking both histidine and tryptophan, 5 mM 3-amino-1,2,4-triazole (3-AT), and 2.5 $\times 10^{-10}$ M E₂. Plates were incubated at 30°C until colonies formed (usually 2–3 days). The plasmids from the colonies were rescued from the yeast cells as described elsewhere (Robzyk and Kassir, 1992), and transformed into *E. coli* DH5 α cells. The plasmids isolated from individual *E. coli* colonies were then re-transformed into the yeast cells and plated on the same selective agar plates again.

For screening randomly picked clones, transformed yeast cells were plated on agar plates containing minimal medium lacking both histidine and tryptophan, and were randomly picked to grow in a 96-well plate. Each well contained 50 μ l minimal medium lacking histidine and tryptophan and the plate was incubated with shaking at 220 rpm and 30°C for 24 h. Five μ l of cell culture from each well was transferred into a fresh 96-well plate. A total of 195 μ l of the same medium containing 5 mM 3-AT and 0.5 μ l ethanol or 10⁻¹⁰, 10⁻⁹, 10⁻⁸ or 10⁻⁷ M E₂ was added into each well. The plate was incubated at 30°C for 48 h and the cell density was measured at 600 nm using a SpectraMax plate reader (Molecular Device, Sunnyvale, CA).

2.7. Growth assays

To quantify the transactivation activity of human ER α mutants, 96-well plates were used to assay the cell growth. Briefly, yeast cells were grown to log phase (OD₆₀₀ 2–5) in 5 ml minimal medium lacking tryptophan and histidine and diluted to OD₆₀₀ 0.02 using the same medium. For 96-well assays, each well contained 200 μ l diluted yeast cells, specified 3-AT concentration, and 0.2 μ l of specified ligand dissolved in 100% ethanol. Plates were incubated at 30°C for the time as indicated and the cell density was measured at 600 nm using a SpectraMax plate reader (Molecular Device, Sunnyvale, CA).

2.8. DNA sequencing

Yeast plasmids were rescued and transformed into *E. coli* DH5 α cells as described in the library screening section. The plasmids from the *E. coli* cells were purified using a

QIAprep spin plasmid miniprep kit (Qiagen, Chatsworth, CA). DNA sequencing was carried out using BigDye™ and an ABI PRISM® 3700 sequencer (Applied Biosystems, Foster City, CA) at the Biotechnology Center of University of Illinois. Primers ERLBD5N and ERLBD3C were used to sequence the mutagenized LBD region of the human ER α genes in both forward and reverse directions.

3. Results and discussions

3.1. Developing the screening method

The screening method was based on the linkage of transactivation activity of human estrogen receptor to the growth rate of yeast cells (Fig. 1). A reporter plasmid 3ERE-pHISi-1 consisting of the yeast *HIS3* gene, driven by the minimal promoter $P_{\min HIS}$ and three tandem copies of estrogen response element (ERE) was integrated into the genome of yeast strain YM4271. The minimum promoter produces a very low level of constitutive expression of the *HIS3* gene, which allows enough cell growth to select for successful integration when constructing the reporter strain.

Human ER α was constitutively expressed from the multi-copy plasmid, YEpER α . YEpER α contains $2\mu ori$ for replication in yeast, and encodes *TRP1* to complement *trp1* defect in the reporter strain. In the presence of ligand, the estrogen receptor forms a dimer and binds to the ERE, which activates high-level expression of the *HIS3* gene, allowing colony growth on minimum medium lacking histidine but containing the 3-AT concentration necessary to eliminate the leaky expression of the *HIS3* gene due to the use of the minimal promoter and the ligand-independent transactivation activity of ER. 3-AT is a competitive inhibitor of the *HIS3* gene product (His3p), which is an essential enzyme in the histidine biosynthesis pathway. During library screening, the transformed yeast cells with ER mutants were grown in minimal medium lacking both histidine and tryptophan, and containing a desired 3-AT concentration and a desired ligand concentration.

To determine the 3-AT concentration required to inhibit background *HIS3* expression, a 3-AT titration experiment was performed both on agar plates and in 96-well plates using the reporter strain YM4271HIS containing YEpER α (Fig. 2). As indicated by the different colony sizes (agar plate assay) and cell densities (96-well plate assay), the

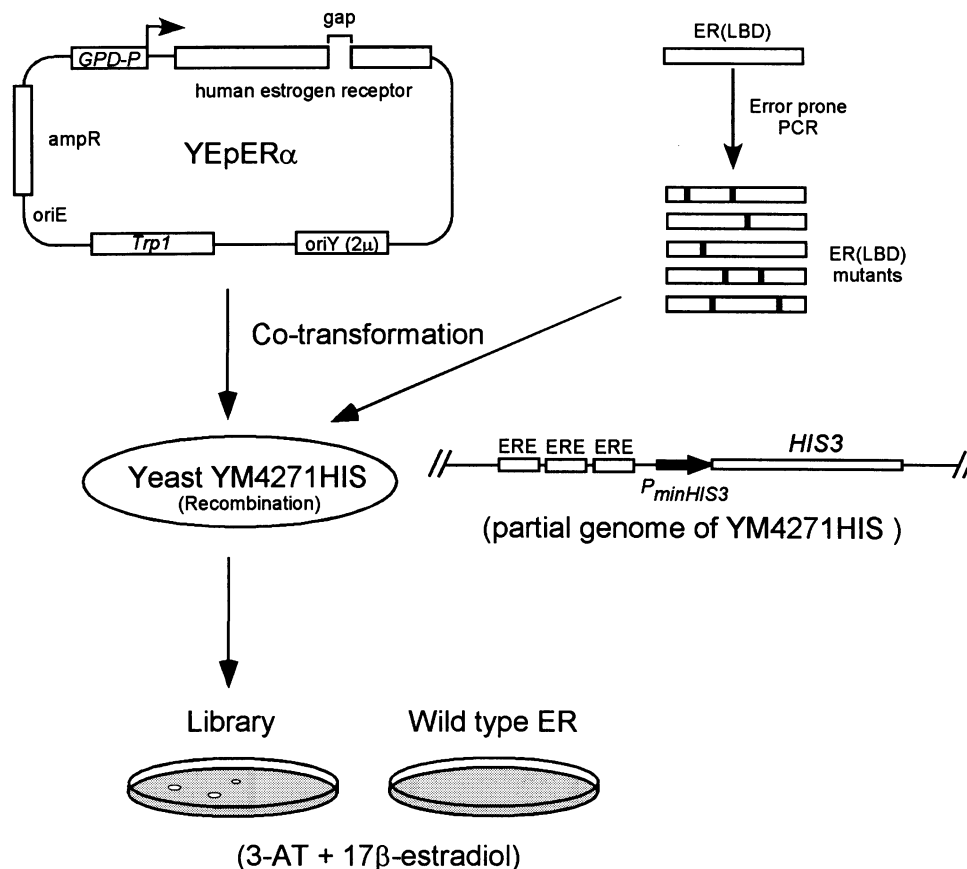


Fig. 1. Schematic representation of the yeast screening method for isolating mutants of human estrogen receptor α with altered transactivation activity. Briefly, a gapped yeast vector YEpER α and randomly mutagenized genes encoding human ER α ligand binding domain were co-transformed into a yeast reporter strain YM4271HIS. Transformants were selected on agar plates containing minimal medium lacking both histidine and tryptophan, 5 mM 3-AT, and a specified concentration of E_2 . Only the human ER α mutants with higher transactivation activity than the wild type human ER α formed individual colonies after incubation at 30°C for 2 days.

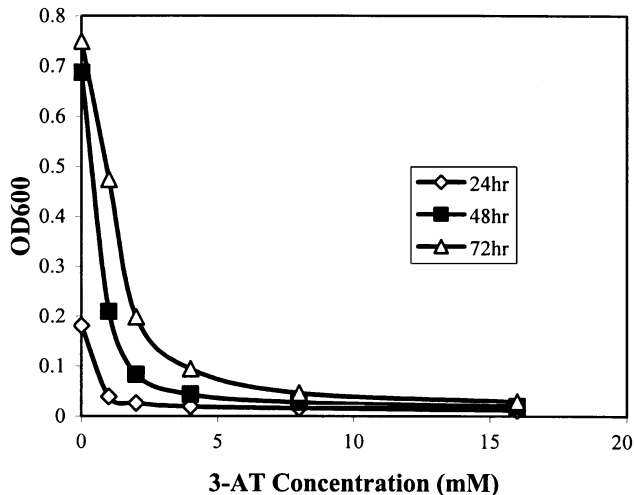


Fig. 2. Response of the wild type human ER α to increasing concentration of 3-AT. The cell density (OD₆₀₀) of yeast cells containing the wild type human ER α was determined using a 96-well plate reader for different incubation time period. No E₂ was added.

yeast cell growth decreased with increasing 3-AT concentration, with the most sensitive region between 0 and 2.5 mM 3-AT. At 5 mM 3-AT, no visible colonies appeared on the selective agar plates even after incubation for 3 days, which allows for stringent selection of those transformants with induced expression of the *HIS3* gene.

To determine the 17 β -estradiol (E₂) concentration that is most sensitive to the altered transactivation activity of human ER α , a similar titration experiment was performed on agar plates using the reporter strain containing YEpER α in the presence of 5 mM 3-AT. Transformed yeast cells were plated on agar plates containing 5 mM 3-AT, various E₂ concentrations or ethanol (negative control), and minimal medium lacking both histidine and tryptophan, followed by incubation at 30°C. It was found that, after 72 h, colonies appeared on plates with 1.25×10^{-9} M E₂, but not on plates with 7.5×10^{-10} M E₂. This observation was consistent with the data from the 96-well plate assay, which showed that the yeast growth rate reached maximum at $\sim 5.0 \times 10^{-9}$ M E₂, and half-maximum at $\sim 1.0 \times 10^{-9}$ M E₂ (Fig. 3). It is noteworthy that the half-maximal concentration (the ligand concentration at half-maximal growth rate) of E₂ for human ER α obtained from the 96-well plate assay was almost identical to that obtained from the standard liquid phase β -galactosidase activity assay as reported elsewhere ($\sim 0.9 \times 10^{-9}$ M E₂) (Wrenn and Katzenellenbogen, 1993), but this half-maximal concentration occurred within a narrower range of E₂ concentration in the 96-well plate-based growth assay than that in the standard liquid phase β -galactosidase activity assay.

3.2. Screening for human ER α mutants with altered transactivation activity

To test if the screening method could be used to rapidly

isolate human ER α mutants with altered transactivation activity, we generated a small library of approximately 10,000 human ER α variants using error prone PCR and yeast in vivo homologous recombination (Fig. 1), and screened them on ten agar plates containing 5 mM 3-AT, 2.5×10^{-10} M E₂, and minimal medium lacking both histidine and tryptophan. The library size was estimated by plating a small portion of the transformants on agar plates containing minimum medium lacking tryptophan. We have chosen to use error-prone PCR to generate random mutations mainly because the mutation rate of error prone PCR can be easily and precisely controlled by the MnCl₂ concentration in the reaction mixture (Zhao et al., 1999). We used 0.15 mM MnCl₂ in the error prone PCR reaction at which concentration it was estimated one to two amino acid substitutions per gene on average was generated (Zhao et al., 1999). Because of its important function in ligand recognition and gene transcription, only the ligand binding domain of human ER α was targeted for random mutagenesis. The re-cloning of mutagenized LBD fragments into the estrogen receptor expression vector was facilitated by the high efficiency yeast in vivo homologous recombination, which allows for the generation of a library of human ER α variants as large as $10^6/(\mu\text{g}$ ligated DNA) without sub-cloning steps in *E. coli*. This DNA cloning procedure is very simple and efficient.

After incubation at 30°C for 3 days, 12 colonies appeared on the selection plates and were picked. To ensure the colony growth was due to the improved function of the estrogen receptor, we isolated the plasmids from these 12 clones and re-transformed them into fresh yeast competent cells as described in Section 2. For each sample, we plated the transformed yeast cells on two selection plates with or without 3-AT and E₂. True positive clones are expected to form colonies on both plates while false positive clones will only form colonies on the plate without 3-AT and E₂. It was

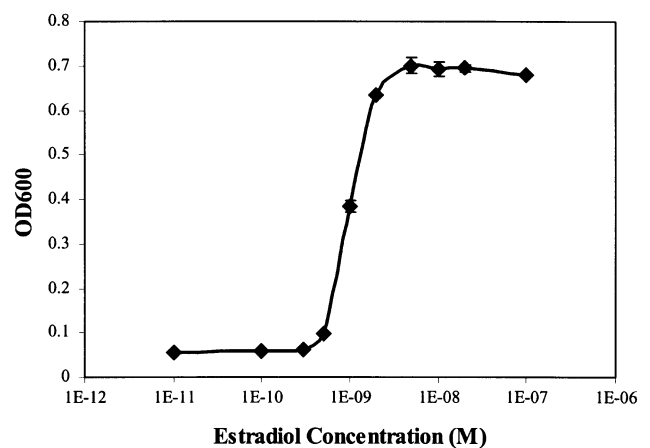


Fig. 3. Dose-response curve of the wild type human ER α to E₂ in yeast cells. The cell density (OD₆₀₀) of yeast cells containing the wild type human ER α to increasing concentrations of E₂ was determined using a 96-well plate reader after incubation at 30°C for 50 h. The cell density was the mean value of two duplicate experiments.

found that two of the 12 clones were false positives (data not shown). Three of the ten positive clones, denoted PC-2, PC-6 and PC-10, were randomly selected for further characterization.

3.3. Characterizing selected human ER α mutants in yeast

To quantify the transactivation activity of human ER α mutants, we assayed the growth of yeast cells expressing three human ER α mutants including PC-2, PC-6 and PC-10, and the wild type human ER α in the presence of ethanol or various concentrations of E₂ in a 96-well plate. As shown in Fig. 4, all three mutants showed increased response to E₂ at the agar plate screening condition compared to the wild type human ER α . Based on its half-maximal concentration, mutant PC-6 was at least 5-fold more sensitive to E₂ than the wild type human ER α . The half-maximal concentrations for mutants PC-2 and PC-10 could not be obtained from the figure because the cells grew too fast even in the absence of E₂. The faster cell growth is likely the result of increased basal level transcription. To overcome this problem, we reduced the initial cell density of these two mutants (OD₆₀₀) to 0.002 in the 96-well plate assay. It was then found that the half-maximal concentration for mutant PC-2 was $\sim 5 \times 10^{-11}$ M E₂ while the half-maximal concentration for mutant PC-10 was $\sim 1.0 \times 10^{-11}$ M E₂ (data not shown). Thus, in comparison to the wild type human ER α , mutant PC-2 was 50-fold more sensitive to E₂ while mutant PC-10 was 100-fold more sensitive to E₂.

To identify the mutations responsible for the altered transactivation activity of human ER α , we sequenced the genes encoding these three mutants. The mutant plasmids were recovered from yeast cells and re-transformed to *E. coli* cells to obtain sequencing quality DNA. Mutant PC-10

has a single mutation K362E, the replacement of a lysine by a glutamate at residue 362. Lysine 362 is located at the C-terminal end of helix 3 in LBD and is conserved in most nuclear receptors. Previous mutagenesis study indicated that this particular residue plays a critical role in ligand-dependent transactivity and co-activator binding (Henttu et al., 1997). Mutant PC-2 also has a single mutation of I358F. Isoleucine 358 is located at the same α -helix as lysine 362, and was identified as one of the three residues forming the hydrophobic patch for co-activator binding in murine estrogen receptor (Mak et al., 1999). Mutant PC-6 has two mutations: K362E and V422M. Both mutations are functional since this double mutant exhibited a different ligand response curve compared to the mutant containing single mutation K362E (mutant PC-2). In fact, V422M should be a deleterious mutation since mutant PC-6 is less sensitive to E₂ than mutant PC-2.

In addition to testing the ability of this screening method to identify mutations for increased transactivation activity, we also tested whether this method can be used to identify mutations that decrease the transactivation activity of human ER α . To this end, we plated a portion of the mutant library on agar containing only minimal medium lacking both histidine and tryptophan. We randomly picked 40 colonies, grew them in a 96-well plate and determined their response curves to E₂. Out of the 40 picked colonies, 27 showed similar growth response to E₂ as the wild type human ER α while 13 showed reduced response to E₂. Three of the thirteen mutants (shown in Fig. 4) were further selected for DNA sequencing. Mutant RC-3 contained a single mutation E353A. This mutation was previously found to cause 100-fold reduction in response to E₂ (Tedesco et al., 2001). Mutant RC-5 contained four point mutations, E353A, T460A, H501R and D545V, which also contained

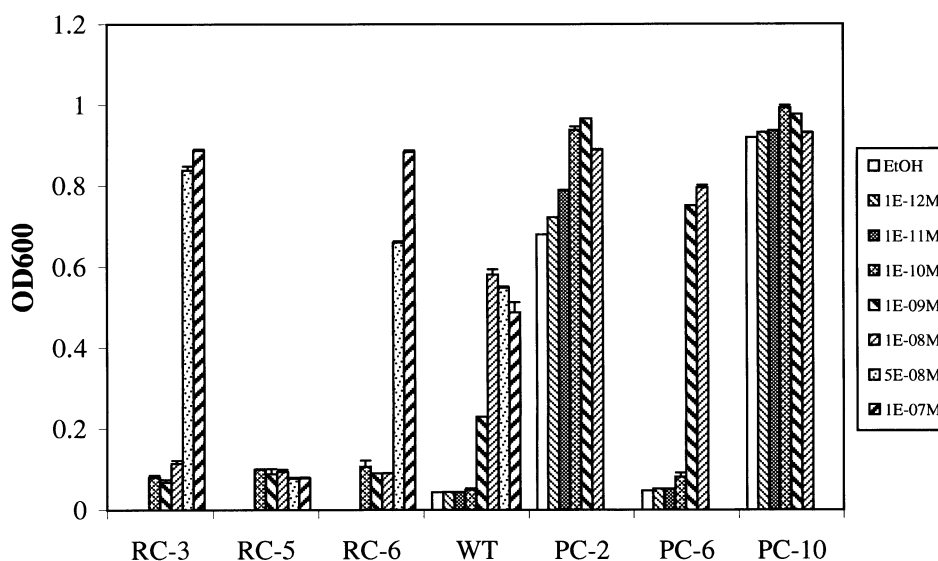


Fig. 4. Comparison of dose-response profiles of the wild type human ER α , three positive human ER α mutants and three randomly picked human ER α mutants to E₂ in yeast cells. The cell densities (OD₆₀₀) of the wild type human ER α and the human ER α mutants to increasing concentrations of E₂ were determined using a 96-well plate after incubation at 30°C for 50 h. The cell density was the mean value of two duplicate experiments.

the same deleterious mutation, E353A. Mutant RC-6 contained a single mutation, I386P. Since residue I386 is located in the middle of helix 6 of LBD and a proline in the middle of α -helix tends to destroy the helical structure, substitution of isoleucine with proline might destroy the structure of helix-6, resulting in a reduced ligand binding affinity of human ER α towards E $_2$.

3.4. Chemical screening

Developing novel estrogen-based therapeutics for human diseases is one of the most important research goals for many academic laboratories and pharmaceutical companies. A rapid and sensitive screening method for identifying

novel estrogenic compounds from chemical libraries should be very useful for such endeavors.

To test this capacity of our screening method, we generated a small library of chemicals consisting of estrogen analogs and non-estrogenic compounds. Yeast cells expressing the wild type human ER α were assayed for growth in 96-well plates that contained 5 mM 3-AT and a series of concentrations of each compound. As shown in Fig. 5A, cells with estrogen analogs could all grow, whereas cells with non-estrogenic compounds including progesterone, hydrocortisone, 20-hydroxyecdysone and 13-*cis*-retinoic acid could not grow even at a very high ligand concentration. Moreover, growth of the wild type human ER α with estrogen analogs generally correlated to their previously

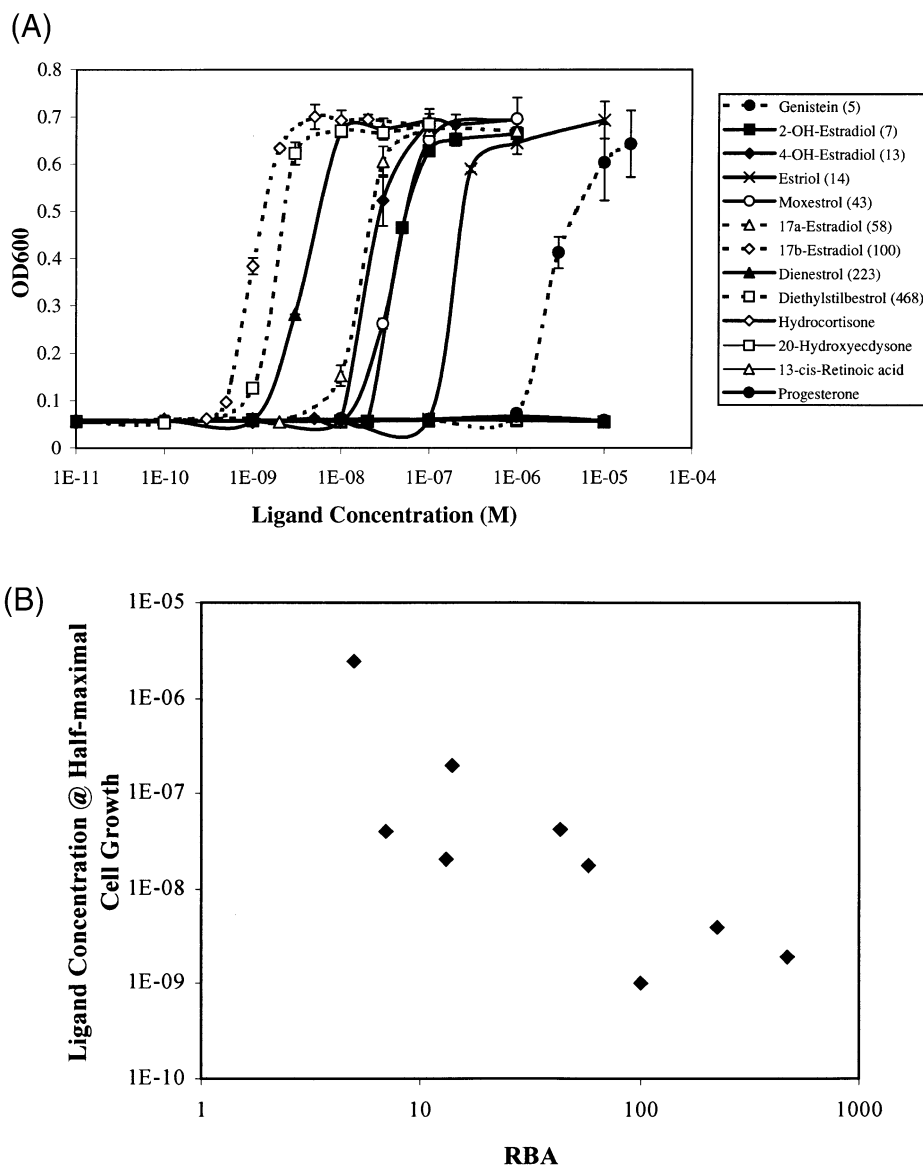


Fig. 5. Chemical screening. (A) Dose-response curves of the wild type human ER α to 13 compounds in yeast cells. OD₆₀₀ was determined after incubation at 30°C for 50 h. The cell density was the mean value of two duplicate experiments. Relative binding affinities (RBAs) for binding of estrogen analogs to human ER α were obtained from Kuiper et al. (1997) and are shown in parentheses. (B) Correlation between RBA and ligand concentration at half-maximal growth rate of yeast cells containing the wild type human ER α based on the results shown in (A). The x-axis is plotted on a logarithmic scale.

measured relative binding affinity (RBA) values to ER α (these RBA values were taken from reference (Kuiper et al., 1997)). Ligands with higher RBA led to half-maximal cell growth at lower ligand concentrations, while ligands with lower RBA led to half-maximal cell growth at higher ligand concentrations (Fig. 5B).

In summary, we have developed a rapid, sensitive and high throughput method for isolating estrogen receptor mutants with altered transactivation activity. Using this method, we have identified several novel mutations in the ligand binding domain of human estrogen receptor responsible for enhanced sensitivity to 17 β -estradiol in yeast cells. We also demonstrated that this method could be used to quantify the trans-activation activity of estrogen receptors, or to screen for novel estrogenic compounds and estrogenic compounds with different binding affinity. In principle, this method might also be used to isolate mutants of any other nuclear receptors for protein structural and functional studies, or identify new therapeutics for nuclear receptor associated human diseases.

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