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RESEARCH

A Bipartite Recombinant Yeast System for the Identification of Subtype-Selective Estrogen Receptor Ligands

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Abstract Since the estrogen receptor α (ERα) and β (ERβ) are thought to mediate different biological effects, there is intense interest in designing or screening subtype-selective ER ligands. Here, we constructed a biosensor identified as bipartite recombinant yeast system (BRYS) to screen and evaluate subtype-selective ER ligands. Uniform design and immunoblotting was used to determine an optimal dose of copper which control the expression of ERs through a copper inducible metallothionine promoter (CUP1). Some chemicals including natural estrogen (17β-estradiol), specific estrogen receptor agonist (PPT and DPN), and phytoestrogens (genistein) were tested to validate this system. There was obvious and anticipative discrimination between the agonistic activities when these chemicals were identified and characterized. Furthermore, antagonist (ICI 182,780), which could antagonize the transactivation of estrogen, and chromatin immunoprecipitation (ChIP) were used to confirm that the agonistic effects were mediated through ERs. Comparative studies showed that this system was reproducible and sensitive. 4.7 pM (1.3 ng/l) estrogen was the lowest concentration that could be detected to ERα and 0.12 nM (33.5 ng/l) for ERβ. The results from our study showed that in vitro screening for subtype-selective ER ligands could be conducted in a simple yeast system that is rapid, sensitive, reliable, and quantitative.

Keywords Bipartite recombinant yeast system · Estrogen receptors · Estrogen response element · Chromatin immunoprecipitation · β-Galactosidase assay

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Abbreviations

AD	Alzheimer's disease
BRYS	Bipartite recombinant yeast system
ChIP	Chromatin immunoprecipitation
CUP1	Copper inducible metallothionine promoter
CYC1	Cytochrome c-1 minimal promoter
ER	Estrogen receptor
ERE	Estrogen response element

Introduction

Estrogen has been known to have various physiological roles in both men and women. In addition to its role in the reproductive system of women, estrogen also plays various roles in cardiovascular health, bone integrity, cognition, and behavior. Given the widespread roles for estrogen, it is not surprising that estrogen is also implicated in the development or progression of numerous disorders, including cancer (breast, ovarian, prostate), neurodegenerative diseases (Alzheimer's disease (AD), Parkinson disease), cardiovascular disease, endometriosis, insulin resistance, obesity, and lupus erythematosus. These diseases can be divided into two groups; those believed to be caused by excessive estrogen, such as breast cancer [1, 2] and endometrial carcinoma [3, 4], and those diseases such as AD [5] that may be relieved by treating with estrogens.

Estrogen replacement therapy for such diseases as AD must take into consideration the wide distribution of estrogen targets and work to minimize potential off-target adverse effects [6]. Interestingly, some substances provide the beneficial effects of estrogen but avoid undesirable side effects. For example, genistein, a major phytoestrogen in soybean, can protect against Aβ-induced death of cultured

SH-SY5Y human neuroblastoma cells without inducing proliferation of uterine endometrial cells [7].

Estrogens control transcriptional responses through binding to two different nuclear receptors, ERα and ERβ, which share a similar architecture [8, 9]. A common amino-terminal region of each receptor plays an important role in target-gene transcription. The central region of both receptors is involved in DNA binding and receptor dimerization. The carboxyl-terminal domain of the receptors is crucial for ligand binding, nuclear translocation, receptor dimerization, and modulation of target gene expression associated with coregulators [10]. The carboxyl-terminal domain between ERα and ERβ shows only 58% homology contrasted to the high similarity of the amino-terminal region [11]. The variability in the carboxyl-terminal domain of ER subtypes is the bases for the respective binding affinities of estrogenic ligands and their variable agonistic transcriptional activities [12]. A previous study showed genistein could selectively bind and activate the ERβ pathways [13]. The tissue-specific effects of estrogenic ligands may result from the distribution of the ER subtypes in different tissues [6].

In an effort to identify subtype-selective ER ligands, we developed a method to screen compounds for their ER subtype specificity. This article describes construction, optimization, and validation of an efficient yeast-based system capable of determining the selectivity of ligands for the ER subtypes.

Materials and Methods

Materials

Estrogen (17β-estradiol), lyticase, and genistein were purchased from Sigma (St. Louis, MO, USA). PPT, DPN, and ICI 182,780 were manufactured by Tocris Cookson, Inc. (Ellisville, MO, USA). 2-Mercaptoethanol and ONPG were provided from Amresco, Inc. (Solon, OH, USA). Antibodies to actin, ERα and ERβ (anti-actin: sc-1616, anti-ERα: sc-7207, anti-ERβ: sc-8974) and Protein A/G agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The goat anti-human IgG was from Zhongshan Golden Bridge Biotechnology, Co., LTD (Beijing, China). Peroxidase-conjugated immunopure goat anti-rabbit IgG (H + L) and supersignal west pico trial kit were purchased from Pierce Biotechnology (Rockford, IL, USA). PVDF membrane was manufactured by Millipore Corporation (Boston, MA, USA). All other chemical supplies were of the analytical grade. We prepared stock solutions of test chemicals in HPLC-analyzed absolute DMSO. Stock solutions were stored at 4°C.

Construction of BRYα and BRYβ

The yeast *Saccharomyces cerevisiae* BJ5409 yeast (MATa, leu2Δ, his3Δ200, urdx-52, trp1, gal) and the YRp2ERE, YEp-hERα and YEp-hERβ plasmids were a gift from Dr. Dan Noonan (University of Kentucky). The YEp-hERs plasmid for expressing human ERα/ERβ controlled by CUP1 promoter contains an ampicillin resistance gene (Ap^r), a tryptophan autotrophy (TRP1) and a 2 micron which facilitate the replication in yeast. The reporter plasmid YRp2ERE carried a uracil autotrophy (URA3), the cytochrome c-1 minimal promoter (CYC1), the reporter gene lacZ encoding β-galactosidase, and two copies of estrogen response element (ERE) upstream of the integrated CYC1 promoter. Schematic maps of these plasmids were kindly provided by Dr. Dan Noonan as shown in Fig. 1. BJ5409 yeast cells were subsequently transformed with YRp2ERE and YEp-hERα or YEp-hERβ according to the TRAFO Protocol [14]. BRYα was constructed by YRp2ERE and YEp-hERα, while BRYβ was constructed by YRp2ERE and YEp-hERβ.

Yeast β-Galactosidase Assay

β-Galactosidase assays were performed essentially as described by Ito [15] and Schultis [16] with some modifications. Upon reaching an OD600 of 0.6, BRYα and BRYβ were incubated in 96-well plate at 30°C for 24 h in the presence of copper ion and chemicals and the OD600 was measured using a Universal Microplate Reader (ELX 800uv, BIO-TEK Instruments, Inc.). Following the incubation period, 100 μl of lyticase solution (Lyticase 1 μg/μl, Z-buffer 1 M (pH 7.0), ONPG 4 mg/ml, SDS 10%) was added to each well, incubated for 2 h at 30°C and OD405 was measured. The β-galactosidase activities were calculated out according to the following formula:

$$\text{Normalized units} = \frac{\text{OD405nm\_sample} - \text{OD405nm\_blank}}{\text{OD600nm\_sample} - \text{OD600nm\_blank}} \times \frac{1}{\Delta t} \times \frac{\text{Vol OD405nm}}{\text{Vol OD600nm}} \times 1000$$

Δt is the incubation time (min) with lyticase at 30°C. Vol represented the volume when the photometric measurement was measured and medium was used as blank and negative control.

Uniform Design

Although copper ion is toxic to the growth of yeast cells, it could induce the expression of ER and the induction is tightly controlled by the concentration of copper ion. We optimized the concentration of copper ion to make the BRYS more efficient. The uniform design method [17, 18]

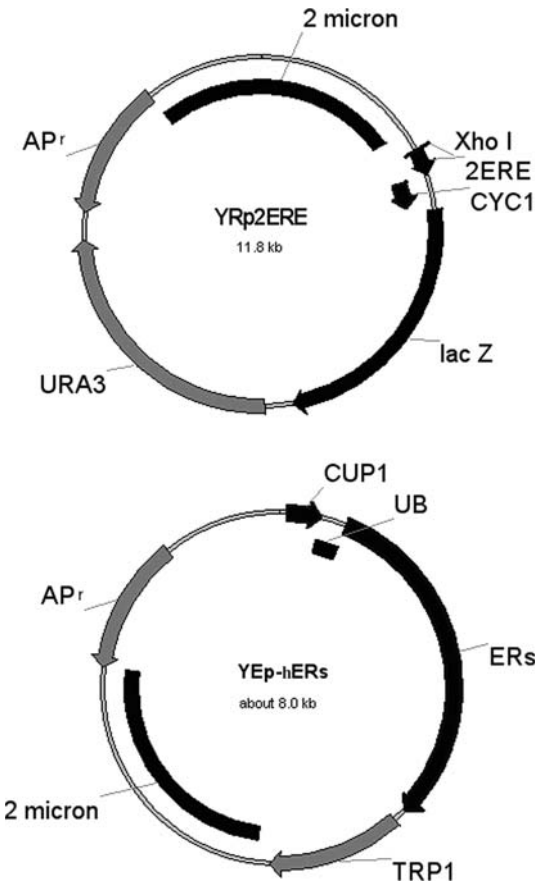

Fig. 1 Schematic drawing of the plasmids used in BRYS. The BRYS consists of a reporter gene plasmid (YRp2ERE) and an expression plasmid (YEP-hERs). The YRp2ERE plasmid contains a uracil auxotrophy (URA3), an ampicillin resistance gene (Ap^r), two copies of the estrogen response element (ERE), the CYC1 promoter and the *lac Z* reporter gene under the control of the ERE. The expression plasmid YEP-hERs carries a tryptophan auxotrophy (TRP1), an ampicillin resistance gene (Ap^r), and the estrogen receptor gene (subtype α or β) controlled by a copper inducible metallothionine promoter (CUP1)

was used to find the best concentration of copper ion. We adopted the $U_{15}(15^7)$ table [19] to explore the dose-dependent relationship between β-galactosidase activities and factors (copper ion and 17β-estradiol). The design of experiments was shown in Table 1.

Immunoblotting

After treated with copper ion for 16 h, the total protein was extracted and its concentration was determined by BCA method. In total, 50 μg total proteins were resolved on 8% SDS-PAGE, transferred to PVDF membrane. The immunodetection was performed with Supersignal West Pico Trial Kit according to the manufacturer's instructions and the PVDF membrane was exposed to Fuji X film. ERs were detected using 0.2 μg/ml rabbit polyclonal antibodies and

Table 1 Uniform design with 15 experiments for two factors at 15 levels, $U_{15}(15^7)$

Group	Factors		X1	X2
	X1	X2	17β-estradiol	Copper ion
1	1	7	−12.0	−9.0
2	2	14	−11.5	−5.5
3	3	5	−11.0	−10.0
4	4	12	−10.5	−6.5
5	5	3	−10.0	−11.0
6	6	10	−9.5	−7.5
7	7	1	−9.0	−12.0
8	8	8	−8.5	−8.5
9	9	15	−8.0	−5.0
10	10	6	−7.5	−9.5
11	11	13	−7.0	−6.0
12	12	4	−6.5	−10.5
13	13	11	−6.0	−7.0
14	14	2	−5.5	−11.5
15	15	9	−5.0	−8.0

Peroxidase-Conjugated ImmunoPure Goat Anti-Rabbit IgG (H + L). Actin was adopted as a loading control.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed according to Hahn Lab' protocol [20] with some modifications. In brief, a single colony of BRYα or BRYβ was inoculated into 5 ml YPD culture and incubated at 30°C overnight, and then 10^{-7} M copper ion was added to induce the expression of estrogen receptors for 8 h. Following exposure to estrogen, yeast cells were fixed with formaldehyde for 20 min, then grinded with acid washed glass beads. DNA was sonicated into small uniform fragments and the DNA/protein complexes were immunoprecipitated using the rabbit polyclonal antibody against estrogen receptor (anti-ERα or anti-ERβ) or the goat antibody against human IgG. Following immunoprecipitation, DNA cross-linking was reversed and proteins were removed with proteinase K treatment. After DNA purification, PCR analysis was performed to measure the enriched amounts of immunoprecipitated DNA. For PCR, 2 μl DNA products were used as templates in 29 cycles of amplification. The primers for PCR amplification of 2ERE were as follows: forward: TCTGAGTTCCGACAACAATG; reverse: GCC ATCCACGCTATATACAC.

Data Analysis

One-way ANOVA was used to detect significant differences between and within replicate experiments. Normality

test was utilized to determine the normal distribution within populations. In the uniform design, all the data were regressed by the Origin 7.0 software (OriginLab, USA). To evaluate the sensitivity, agonistic potency, and efficiency, we empirically derived a dose-related equation from a number of nonlinear regression models for final data analysis.

We used the Sigmoidal Equation (Boltzmann Model):

$$Y = \frac{A2 - A1}{1 + e^{(x-EC50)/dx}} + A1$$

Variables A1 and A2 are the minimal and maximal observed β-galactosidase activities. X is the concentration of test agent; EC50 is the concentration of test agent yielding half-maximal β-galactosidase activities and dx is the slope parameter.

Results

Construction of BRYS by Transformation of the Reporter Gene Plasmids and the ERs Expression Plasmids

BJ5409 yeast cells were subsequently transformed with the reporter gene plasmids (YRp2ERE) and the ERs expression plasmids (YEp-hERα or YEp-hERβ). BRYα was constructed by YRp2ERE and YEp-hERα, while BRYβ was constructed by YRp2ERE and YEp-hERβ. Under the induction of copper ion, ERα or ERβ could be expressed. Estrogen and ERs could form estrogen-ERs complexes which could be recruited to ERE. Subsequently, the transcription of *lac Z* reporter gene which encoded β-galactosidase was initiated. By measuring the β-galactosidase activity in lysates of the transformed yeast cells, the estrogenic effects of different ligands could be analyzed. Since BRYα or BRYβ contained only one kind of ER (ERα or ERβ), so the ER subtype selectivity of the ligands could also be represented.

Copper Ion Induction of Estrogen Receptor Expression

In this system, the copper ion responsive CUP1 promoter regulates the expression of ERs in a dose-dependent manner. Even though the CUP1 promoter contributes to copper resistance and copper detoxification [21], a high dose of copper ion is still toxic to the BJ5409 yeast strain. Cytotoxicity is negligible when copper ion concentration is below 10^{-5} M (data not shown). To well study the influence of the concentrations of estrogen and copper ion on the BRYS, uniform design methodology was used. Uniform design methodology, referred as Uniform Design Experimentation, is an experimental design method. It is an efficient fractional factorial design and has been recognized as an important space-filling design. It could investigate two or more factors together in an experiment just like orthogonal experimental design. In our study, we considered two factors: estrogen and copper ion together. In the range of 10^{-5} and 10^{-12} M, we took representive concentrations of estrogen and copper ion which were uniformly distributed and measured the β-galactosidase activities under these selected concentration. The $U_{15}(15^7)$ strategy (Table 1) was used to determine the optimal concentration of copper ion and estrogen as measured by β-galactosidase activity. In the $U_{15}(15^7)$ strategy, estrogen or copper ion concentrations from 10^{-5} to 10^{-12} M were split into 15 levels. Fifteen groups were treated with estrogen and copper ion respectively at indicated levels as shown in Table 1. For example, in group 1, the first level of estrogen (10^{-12} M) and the seventh level of copper ion (10^{-9} M) were coincubated in this biosensor and the β-galactosidase activities were measured. The β-galactosidase activities at different combinations of copper ion and estrogen (both ranging from 10^{-5} to 10^{-12} M) were shown in two 3D diagrams (Fig. 2). Both estrogen and copper ion could alter β-galactosidase activities. The induction of estrogen was dose-dependent. There was a plateau between 10^{-7} and 10^{-5} M and an oblique plane between 10^{-10} and 10^{-7} M estrogen (Fig. 2a). Copper ion concentrations also affected the β-galactosidase activities (Fig. 2b). When the copper ion concentration was under 10^{-10} M, the β-galactosidase activities began to decrease. To make this system much more sensitive, the concentrations of copper ion must be higher than 10^{-10} M. Besides, when 10^{-7} M estrogen was co-incubated with different copper ion concentrations (10^{-6}, 10^{-7}, and 10^{-8} M), there was no difference in β-galactosidase activities ($P < 0.05$) (data not shown). The same results were also observed with BRYβ (data not shown).

Immunoblotting with anti-ER antibodies was used to confirm copper ion induction of each ER subtype. The results showed a dose-dependent induction of ERs expression following exposure to four different copper ion concentrations (10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M) (Fig. 3). The expression of ERs was undetectable when no copper ion was added. With the increase of copper ion concentrations, the ERs levels increased (10^{-10} M) and got to high levels at 10^{-8} and 10^{-6} M. In combination with the uniform design results, we selected a final concentration of 10^{-7} M copper ion to induce the expression of ERs in all the subsequent experiments.

Characterization of BRYS with Known Ligands

The agonist actions of estrogen, PPT, DPN and genistein were measured. Estrogen could promote the β-galactosidase

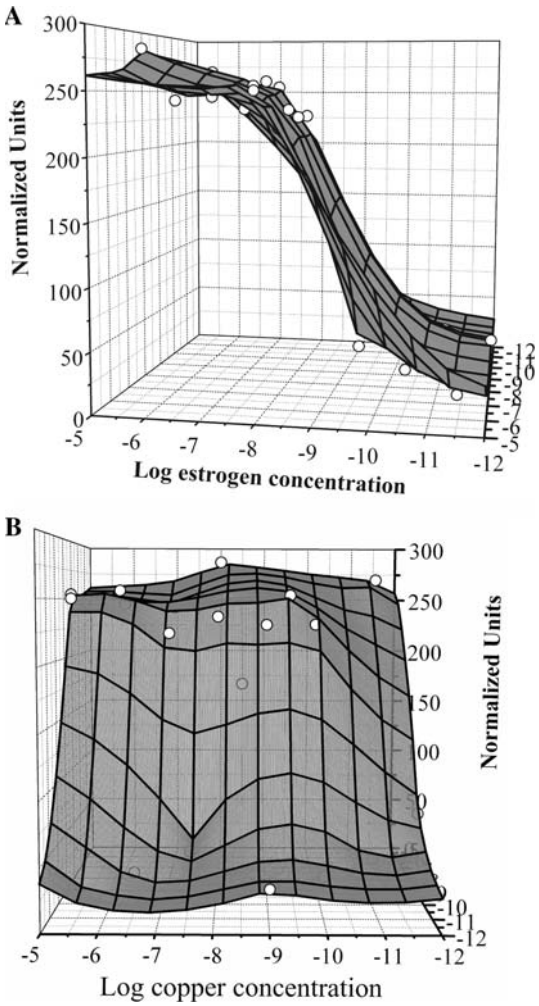

Fig. 2 Effects of copper ion and estrogen on the induction of β-galactosidase activities. With the aid of $U_{15}(15^7)$ (Table 1), the β-galactosidase activity at different combination of copper ion and estrogen concentrations from 10^{-5} to 10^{-12} M was reported. (**a**) Influence of estrogen on the β-galactosidase activity There was a plateau between 10^{-7} and 10^{-5} M and an oblique plane between 10^{-10} and 10^{-7} M estrogen. (**b**) Copper ion also altered the β-galactosidase activity at a dose-dependent manner

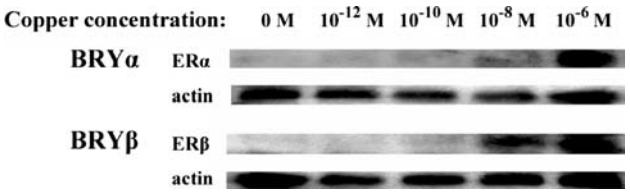

Fig. 3 The ER proteins were induced by different concentration of copper ion. After the treatment of 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M copper ion for 16 h, the yeast cells were harvested and analyzed by immunoblotting with anti-ER antibodies and anti-actin antibody. Copper ion regulated the expression of estrogen receptors dose-dependently. The expression of ERs was undetectable when no copper ion was added. With the increase of copper ion concentrations, the ERs levels increased (10^{-10} M) and got to high levels at 10^{-8} and 10^{-6} M

expression in BRYS with a marginally higher efficiency in BRYα than in BRYβ (Fig. 4a). A mean value of 0.18 ± 0.01 nM for the EC50 of BRYα, and 0.49 ± 0.05 nM for the EC50 of BRYβ were obtained. To allow comparison of subsequent analyses, the potency and efficiency of estrogen were set as 100. The relative potency was calculated from $100 \times EC50_{(estrogen)}/EC50_{(ligand)}$ and the relative efficiency was calculated from $100 \times (A2 - A1)_{(ligand)}/(A2 - A1)_{(estrogen)}$.

PPT and DPN are subtype-selective ER ligands. In the transcription assays, PPT (Fig. 4b) and DPN (Fig. 4c) displayed obvious ER subtype selectivity. PPT showed ERα preference. The ratio of relative potency (β:α) was 0.67 and the ratio of relative efficiency (β:α) was 0.36 (Table 2). In contrast, DPN demonstrated ERβ selectivity with a 21.57-fold ERβ potency selectivity. Phytoestrogen genistein, derived from a number of plants, displayed obvious ERβ selectivity in our yeast-based system (Fig. 4d). The EC50 for BRYα was 4.64 ± 0.88 μM and 4.66 ± 1.13 nM for BRYβ. Genistein showed strong ERβ potency selectivity with a β:α ratio of 2710.54 (Table 2).

The Transactivation of Estrogen could be Antagonized by ICI 182,780

The compound ICI 182,780 has been reported to be a pure estrogen receptor antagonist [12]. The co-incubated of 17β-estradiol and ICI 182,780 in either BRYα or BRYβ showed a dose–response relationship in the concentration range tested (Fig. 5). In agreement with Wang's and Bovee's results [22, 23], ICI 182,780 at a high concentration showed decreased β-galactosidase activity. The inhibitory effects were even more obvious in our system than that in Bovee's results. Regression analysis of the data to sigmoidal formula, an IC50 value of 0.93 ± 0.06 μM for BRYα and 0.28 ± 0.02 μM for BRYβ were calculated. The inhibitory effects observed with ICI 182,780 in BRYS support the notion that the agonistic effects of the compounds tested is through their interaction with estrogen receptors [8].

Occupancy of ERE by ERs After the Induction of Estrogen

ChIP assay was used to determine if the ERE of YRp2ERE was occupied by ERs after estrogen treatment. The results showed that the occupancy of ERE by ERs was temporary following induction by estrogen exposure. Figure 6b shows ERE occupancy after 0, 5, 15, 25, and 35 min of estrogen treatment with ERE occupancy detected after 25 min of estrogen exposure. No occupancy was observed when immunoprecipitation with IgG was performed (Fig. 6c). Shang found that ER and a number of coactivators rapidly

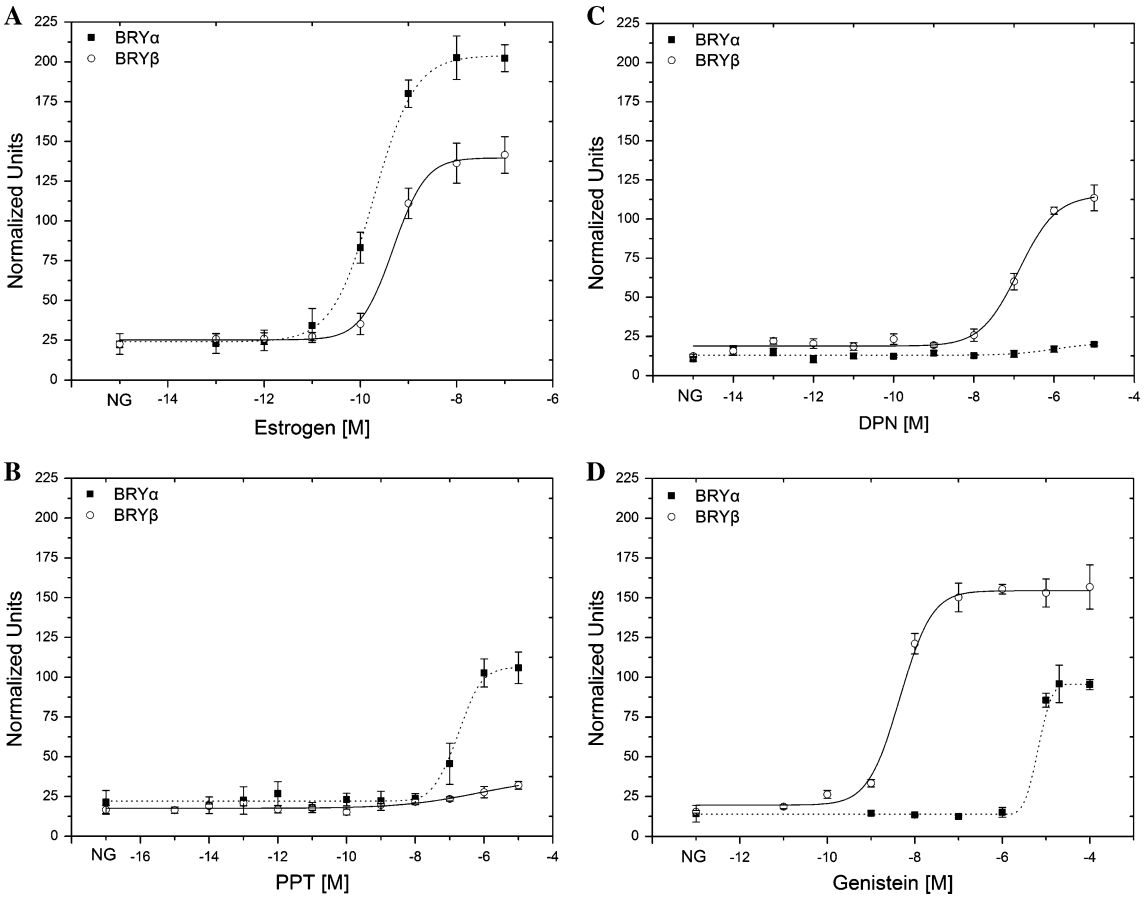

Fig. 4 Agonistic effects of four well-studied chemicals including estrogen, PPT, DPN, and genistein. BRYα or BRYβ were co-incubated with one of the specified chemicals at the concentrations indicated for 24 h, and then the β-galactosidase activities were measured. The β-galactosidase activities of BRYS without induction of estrogenic chemicals were set as negative control (NG). Data represented as mean ± SD (n = 3–9)

Table 2 The EC50 values, relative potency, and relative efficiency, based on the BRYS results were given for the chemicals tested

Ligands	EC50		Relative potency[a]			Relative efficiency[b]		
	ERα	ERβ	ERα	ERβ	β:α	ERα	ERβ	β:α
Estrogen	0.18 ± 0.01 nM	0.49 ± 0.05 nM	100	100	1	100	100	1
PPT	0.17 ± 0.03 μM	0.69 ± 0.16 μM	0.11	0.071	0.67	46.7	17.0	0.36
DPN	1.03 ± 0.17 μM	0.13 ± 0.01 μM	0.017	0.38	21.57	4.3	84.5	19.65
Genistein	4.64 ± 0.88 μM	4.66 ± 1.13 nM	0.0038	11	2710.54	45.7	118.5	2.59

Note: Sigmoidal regression was taken by Origin 7.0

[a] Relative potency = $100 \times EC50_{estrogen}/EC50_{ligand}$

[b] Relative efficiency = $100 \times (A2 - A1)_{ligand}/(A2 - A1)_{estrogen}$

associated with estrogen responsive promoters following estrogen treatment in a cyclic fashion. Cycles of ER complex assembly were followed by gene transcription [24]. Twenty-four hours later, several cycles have been taken place and then we could measure the calculative β-galactosidase activity. The ChIP findings demonstrate that in yeast estrogen is capable of activating ERs to bind ERE and initiate transcription. Furthermore, these results suggest the ER subtype selectivity of the different ligands may be due to the difference in their respective ability to bind to the ERE.

Reliability of BRYS

The reliability of the BRYS is dependent on its reproducibility and limit of detection. For reproducibility, three

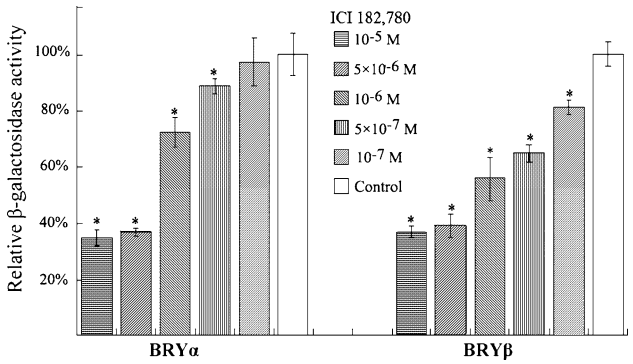

Fig. 5 Inhibition of estrogen by ICI 182,780. BRYα and BRYβ were treated with 10^{-7} M estrogen and ICI 182,780 at indicated concentration for 24 h, and then the β-galactosidase activities were measured. The induction by estrogen alone without ICI 182,780 was determined as control (100%). *Statistically significant difference ($P < 0.01$)

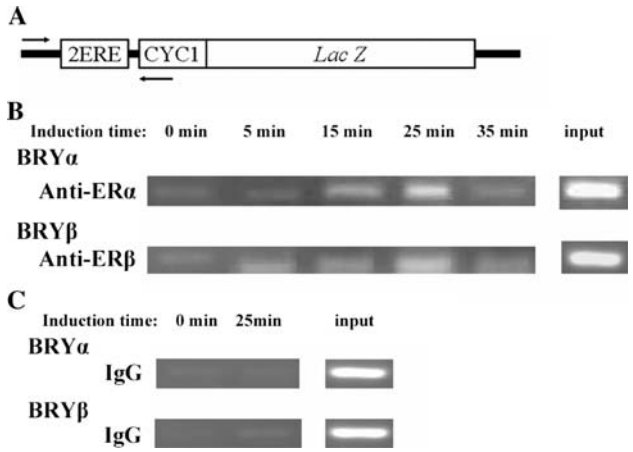

Fig. 6 Validation of the occupancy of ERs to ERE by ChIP. (**a**) Amplified region by ChIP was shown. (**b**) The yeast cells were treated with 10^{-7} M estrogen for 0, 5, 15, 25, and 35 min. The target DNA/protein complexes were immunoprecipitated using the rabbit polyclone antibody against estrogen receptor (anti-ERα or anti-ERβ), then semi-quantitative PCR of inputs and immunoprecipitation outputs were performed. (**c**) Estrogen (10^{-7} M) was used to induce BRYS for 0 and 25 min, and then target DNA/protein complexes were immunoprecipitated using the rabbit polyclone antibody against IgG. Semi-quantitative PCR of inputs and immunoprecipitation outputs were performed

assays with one specimen were repeatedly performed on three separate days. The reproducibility was assessed by A1, A2, dx, and their standard deviations. The limit of detection was defined as a change of three times the standard deviation of the negative control plus the mean value of the negative control.

A standard dose–response curve with estrogen was generated and fitted to a sigmoidal equation. The parameters A1 and A2 were calculated for both BRYα and BRYβ (Fig. 7). Normality tests showed the populations of A1 and A2 are normally distributed ($\alpha = 0.05$). One-way ANOVA

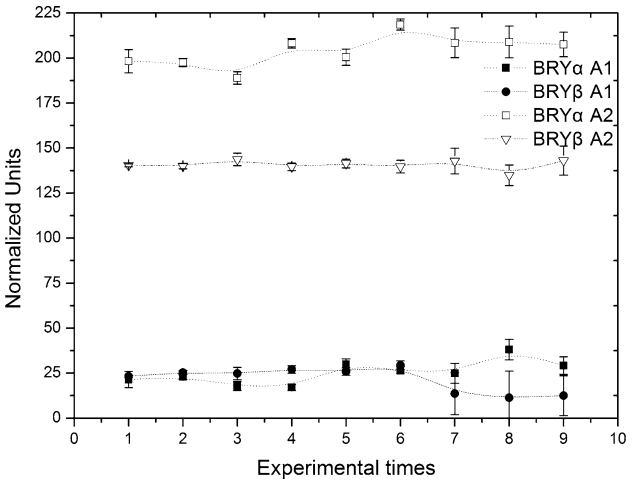

Fig. 7 Parameters for sigmoidal curves of estrogen. Parameter A1 and A2 were plotted. At the 0.05 level, the populations of A1 and A2 were normally distributed. Data represented as the mean ± SD for three samples

indicated A1 values between ERα and ERβ were not significantly different ($P < 0.05$). For BRYα, mean value of 22.8 ± 0.5 for A1, 203.6 ± 2.0 for A2, and 0.4 ± 0.02 for dx were obtained. For BRYβ, mean value of 26.1 ± 0.7 for A1, 139.9 ± 1.5 for A2, and 0.29 ± 0.03 for dx were calculated out. For both BRYα and BRYβ, the standard deviation (SD) of each parameter in the sigmoidal equation was small. The BRYS system was thus considered reproducible and acceptable for large-scale screening and evaluation assays.

The limit of detection of the bioassay was tested by serial dilutions of estrogen ranging from 10^{-13} to 10^{-7} M (BRYα and BRYβ) with buffer as the negative control (Fig. 4a). The results indicated that 4.7 pM (1.3 ng/l) or 0.12 nM (33.5 ng/l) estrogen was the lowest concentration which could be detected in BRYS with ERα or ERβ expressed.

Discussion

In this study, we constructed a yeast-based biosensor to screen and evaluate ER subtype selective agonists. Optimization and validation were conducted to make this system sensitive, stable, and reproducible. This biosensor included a reporter gene plasmid (YRp2ERE) and a ERs expression plasmid (YEp-hERα or YEp-hERβ). After addition of 10^{-7} M copper ion, ERα or ERβ could be tightly regulated and expressed at an appropriated level. When this biosensor was applied to screen and evaluate ER subtype selective agonists, those chemicals were incubated with the recombinant yeast cells with ERα or ERβ expressed. If the chemicals were estrogenic, it could form ligand-ERs complexes with ERs. After dimerization, the

dimer of ligand-ERs complexes could be recruited to the enhancer (ERE). Subsequently, the transcription of *lac Z* reporter gene which encoded β-galactosidase was initiated. We could measure the estrogenic effects of different ligands by measuring the β-galactosidase activity in lysates of the transformed yeast cells. Since the BRYα or BRYβ contained only one subtype of ERs (ERα or ERβ), we could test the agonistic effects of the ligands and evaluate the ER subtype selectivity of the ligands.

The results of the study demonstrated the system was well-designed. CUP1 promoter is the metalothionein gene promoter and can be used to make a gene inducible by copper ion added to the medium. Under copper-limiting conditions the basal level of expression from the CUP1 promoter is very low [25]. But after applying the copper ion, the activation of target genes under CUP1 promoter is rapid, tight, and robust [26]. Copper is toxic to the yeast cell (BJ5409 strain), high dose of coper ion can inhibit the growth of yeast cells. By using uniform design and Immunoblotting, an optimal copper ion concentration (10^{-7} M) was determined.

After optimization, validation of this system with well-known ligands including estrogen, PPT, DPN, and genistein was performed. The EC50 values, relative potencies, and relative efficiency of the four ligands tested are shown in Table 2. To achieve equivalent amount of induction, each ligand required a different concentration. When determining the subtype selectivity of an estrogenic ligand, it was important to consider EC50 value, relative potency, relative efficiency, and other characters such as relative binding affinity and engagement of coregulators. For example, estrogen showed no ER subtype selectivity based on similar EC50 values even though the efficiency to ERα was slightly different from ERβ.

PPT, on the other hand, preferentially activated ERα. The ratio of relative efficiencies and potencies between BRYα and BRYβ was nearly 3-fold and 1.5-fold, respectively. DPN was shown to be ERβ selective agonist. The β:α ratios in relative potencies of DPN was 21.57. The ER subtype selectivity of both PPT and DPN was lower than those in 293 human embryonal kidney cells [27] or HELN cell line stably expressing ERα or ERβ [28]. Engagement of coregulators present in mammalian cells may make the ER subtype selectivity more obvious.

Despite the differences of coregulators expression within those cell types, the results from these systems are comparable. It is most likely that the compound and the ER subtype are the major factors to determine the agonistic or antagonistic effects. Thus, it is expected that the application of the BYRS will yield important and useful information on estrogenic ligand subtype selectivity.

The dose-dependent inhibition of antagonist and ChIP technology proved that the agonistic effects of ligands were mediated through ERs. The binding affinity of a ligand to the different ERs and the conformation of ligand-ER-ERE complex are able to affect the estrogenic activity and subtype selectivity. When both of these variables are suitable, the agonistic effect of a ligand is detectable. It is likely that selective estrogenic chemicals can induce the different ERs to bind to ERE and the suitable conformation of ligand-ER-ERE complex is capable of activating transcription. The ER subtype selectivity of chemicals might be explained by the differences in inducing ER subtypes to bind to ERE and in initializing the gene transcription.

Previous study of Shan's [24] has shown that ERs and coactivators rapidly associated with estrogen responsive promoters following estrogen treatment in mammalian cells. The binding of ERs to estrogen responsive promoters is in a cyclic fashion, each followed by estrogen responsive gene transcription. In Shan's study, ER binding peaked at 45 min in MCF-7 cells. However, in our study, both ERα and ERβ binding peaked at 25 min (Fig. 6b). It demonstrated that ER activation is also short-lived in yeast cells. It is interesting that the response in yeast cells (25 min) was faster than in mammalian cells (45 min) and the mechanism was unknown.

Compared with other yeast assays that have been described, most of them have not considered the transcriptional activities of both ERα and ERβ. Previous assay systems have focused on the human ERα [29]. Our BRYα and BRYβ system, in contrast, can be transformed by either ERα or by ERβ, respectively, allowing us to discriminate whether a chemical is ERα or ERβ reactive. This unique advantage makes the yeast system superior to mammalian cell system given that, sometimes, only one kind of receptor can be expressed in a mammalian cell line [28]. So the differences of estrogenic activities of chemicals reacting with ERα or ERβ could just be well studied in the yeast-based bioassays which lack any of the receptors originally. In addition, the toxicity of test agents is a potential problem. As cytotoxicity occurs more frequently in mammalian cell assays than yeast-based assays, the yeast cell is more resistant to heavy metals and endotoxins [30]. Thus yeast assays can be a better method to screen and evaluate estrogenic chemicals and ER subtype selective ligands.

The detection limit of the system is excellent since a concentration of as low as 4.7 pM 17β-estradiol can be detected in the BRYα system. This can be compared to the almost 25-fold high sensitivity achieved in a similar system established by Jungbauer [29] to detect estrogenic activity with ERα. Thus, our system is particularly useful for detecting low concentrations of active compounds, especially those extracted from limited amounts of material. With the system described here it takes just 2 days to complete an entire assay with a large number of samples.

Now we achieved a sensitive, reliable, inexpensive, and high throughput screening system for the determination of estrogenic agonistic activity of unknown samples. The system can also easily be extended to other steroid hormone receptors.

Conclusion

The purpose of constructing the BRYS systems was to develop a model to discriminate and characterize the ER subtype selectivity of chemicals. We introduced either ERα or ERβ into the yeast system combined with a reporter plasmid composed of two copies of ERE upstream of the integrated CYC1 promoter. After optimization and validation, the agonistic effects of the test chemicals and to which receptor they preferred to activate were assessed by measuring the β-galactosidase activity. This model was proved to be sensitive, reliable, and quantitative.

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