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Development of a simple screening system for endocrine disruptors

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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: An endocrine disruptor is a synthetic chemical, which causes adverse effects in an organism, or its progeny, after causing perturbations in the endocrine system. It is important to know which synthetic chemicals have endocrine-disrupting action. However, an increasing number of synthetic chemicals are being produced by modern synthetic chemistry, and the examination of endocrine disruptor potential has not yet caught up with the advances in synthetic chemistry. In this study, we have developed such a screening system for detecting synthetic chemicals with estrogen-like effects.

Material/Methods: The system was based on the yeast one-hybrid system. Both *HIS3* and *lacZ* reporter genes connected to three tandem copies of the estrogen response element were prepared. Gal4-estrogen receptor is a fusion protein made from the activation domain (AD) of the yeast GAL4 transactivator gene and then incorporated into a plasmid, which was transfected into the YM4271 yeast cell strain. The estrogen effect was judged by this developed screening system.

Results: A dual reporter assay-system was established by transfection of the both *HIS3* and *lacZ* reporter genes into the yeast cells. This screening system enabled the detection of as little as 10^{-12} mol of β-estradiol.

Conclusion: These results show that this newly developed dual assay is useful for the screening of endocrine-disruptors that have estrogen-like action.

key words: **endocrine disruptors • estrogen • one-hybrid system • synthetic chemicals • screening system • estrogen receptor**

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BACKGROUND

Recently, much interest has been shown in endocrine disrupters as environmental pollutants [1,2]. An endocrine disruptor is a synthetic substance that can cause adverse effects in organisms or their progeny following the disruption of endocrine system. Disturbances of steroid hormone production in the gonads have become a serious problem because of estrogen-like chemicals cause reproductive dysfunction [3]. It has been reported that diethylstilbestrol (DES), a synthetic estrogen prescribed to pregnant women to prevent spontaneous abortion, caused an increased risk of vaginal cancer in the female progeny of women taking DES [4]. There have been reports of prostatic, testicular cancers and cryptorchism in males who have been exposed to high doses of estrogen [5–7], and a fifty percent reduction in human sperm counts has also been reported in the last fifty years [8].

Steroid hormones combine with receptors and the receptor-DNA complex then enters the nucleus. The mechanism of action of the endocrine disruptors is initiated by the binding of the chemical to the nuclear receptor. Some chemicals act with a similar hormonal function as the organism, while other chemicals present inhibit hormonal functions. The chemicals with estrogen-like actions include: nonylphenol or bisphenol A [9,10]. The estrogen-like effect is caused when chemicals combine with the estrogen receptor (ER). DDE or Vinclozolin are examples of androgen competitors that bind to the androgen receptor [11] and inhibit the androgen hormone activity.

There are about seventy chemicals that are thought to have endocrine-disrupting effects. It is important to know which synthetic chemicals have endocrine-disrupting action and to what degree the endocrine systems of organisms are affect. However, an increasing number of synthetic chemicals are being produced by modern synthetic chemistry, and the examination of endocrine disruptor potential has not yet caught up with the advances in synthetic chemistry. It is therefore necessary to develop a screening test for detecting synthetic chemicals that have an endocrine-disrupting potential. A system for determining the severity of endocrine-disturbance is also needed. In this study, we have developed such a screening system for detecting synthetic chemicals with estrogen-like effects.

MATERIAL AND METHODS

Principles

Many transcription factors are composed of a DNA-binding domain (DNA-BD) and an activation domain (AD). The DNA-BD and the AD form a complex, which results in the downstream gene transcription. The one-hybrid system is an assay that enables the identification of interaction between DNA and proteins *in vivo* using the yeast host [12,13]. When an AD fusion protein combines with the bait arrangement, an *HIS* reporter gene is transcribed and the yeast can grow on a colony with minimal culture medium without histidine. In our screening system, a reporter gene was constructed by the insertion of estrogen response elements (ERE) upstream of the reporter gene (*HIS3* or *lacZ*), which is integrated into the reporter yeast genome. *HIS3* or *lacZ* is activated in the reporter gene when ER is fused with AD in the presence of an estrogen-like molecule. Then, a colony can grow on the minimal culture medium lacking histidine. Interaction of the ER with DNA can also be detected using the *lacZ* expression with the β-galactosidase enzyme.

Yeast strain

We used the yeast strain YM4271 (CLONTECH Laboratories, Inc, Palo Alto, CA). YM4271Genotype: MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-Δ512, gal80-Δ538, ade5::hisG.

Plasmid Constructs

pHISi is an *HIS3* reporter gene designed to be integrated into the yeast *HIS3* gene locus (CLONTECH Laboratories, Inc.). pLacZi is a *lacZ* reporter gene designed to be integrated into the *lacZ* gene locus. To prepare the ERE, two oligonucleotides (5'-GATCCGGGTCACAGTGACCGCTAG-3' and 5'-GATCTCCTAGCGGTCACTGTGACCC-3') were synthesized using a DNA synthesizer. The sense oligonucleotide has a *Bam*HI endonuclease recognition sequence, and the antisense oligonucleotide has a *Bgl*II endonuclease recognition sequence. Both the sense and antisense oligonucleotides of the ERE were heated at 90°C for three minutes and at 65°C for 10 minutes. They were then incubated at 37°C for 10 minutes and cooled to 4° to form the double-strand DNA. The double-strand DNA was then separated and refined by 1% agarose gel electrophoresis. The double-strand ERE was then inserted into the *Bam*HI/*Bgl*II site of the pTK vector (pTK1×ERE). To make two tandem copies of ERE, pTK1×ERE was treated with *Bgl*II and *Nde*I. In addition, pTK1×ERE was also cut with *BamH*I and *Nde*I. The *Bgl*II/*Nde*I-cut small-fragment and a *BamH*I/*Nde*I-cut large fragment were then ligated. A pTK2×ERE vector that has two tandem copies of the ERE was thus prepared. To make three tandem copies of the ERE, pTK2×ERE was cut with *Bgl*II/*Nde*I and pTK1×ERE was cut with *BamH*I/*Nde*I, and they were ligated to each other. pTK3×ERE was cut with *BamH*I/*Bgl*II, and three tandem copies of the ERE were processed by T4 polymerase to made blunt-ended termini. pHISi or pLacZi were digested with the *Sma*I endonuclease to prepared 5'- or 3'-blunt terminal ends. Three tandem copies of the ERE were ligated to the pHISi or pLacZi reporter plasmids, and pLacZi3×ERE and pHISi3×ERE having three tandem copies of the ERE were made. A plasmid expressing a GAL4-ER fusion protein (pGAD424ER) was constructed by inserting an *Eco*RI fragment prepared by PCR using ERα cDNA as a template into the pGAD424 vector. The plasmids were prepared for transfection studies using a Qiagen Maxiprep system (QIAGEN, Hilden, Germany).

Integration of the ERE reporter constructs into the yeast genome

The pHISi3×ERE was digested with *Xho*I and pLacZi3×ERE was digested with *Nco*I to linearize the plasmid constructs. The reporter constructs were transformed with the pHiSi3×ERE into the YM4271 yeast strain using the lithium acetate method according to the recommended protocol of the manufacturers (Yeast Transformation System, CLONTECH Laboratories, Inc.). Transformants were then placed on to a synthetic dropout (SD) lacking histidine selection medium for *HIS3* expression strains. Then, we transfected a linearized pLacZi3×ERE expression vector into the yeast integrated *HIS* reporter construct. The pLacZi transformants were then plated on SD/-His/-Ura plates to select colonies with an integrated *Ura3* reporter construct and incubated at 30°C for six days. Competent *HIS3* and *lacZ* expression cells were transformed with the GAL4AD ER fusion constructs (pGAD424ER) and the transformants were placed on to medium lacking His and Ura and Leu (SD/-His/-Ura/-Leu) in order to select for GAL4 activating domain. 3-Aminotriazole (3-AT) was added to the medium at a concentration of 30 mM to prevent the growth of colonies due to leaked histidine expression.

Estrogen effect judgment

We plated the established yeast strain (100 µl stock) on the agar plate containing selection medium SD/-His/-Leu/-U+30mM 3-AT. One hundred µl of various concentrations of chemicals in ethanol were spread onto the center of the plate. The plates were incubated at 30°C for four days until well good grown colonies with the *HIS3*, *lacZ* and *ER* transcripts were expressed in the yeast strain. Colonies that grew on the selection medium (SD/-His/-Ura/-Leu) were considered to demonstrate an interaction between a GAL4 ER fusion protein and ERE that allowed His production in yeast cells after transactivation of the *HIS3* reporter. When the size of the yeast colony on the plate was over 2mm, then the estrogen effect was judged as positive. To verify the ERE and ER interaction, β-galactosidase assays were also performed. A β-galactosidase filter assay was used for determining β-galactosidase activity according to the Yeast Protocols Handbook (CLONTECH Laboratories, Inc.). Colonies were lifted on to Whatman no. 5 filter papers and cells were permeabilized by a freezing-thawing cycle in liquid nitrogen and then placed on a filter presoaked in X-Gal solution and incubated at room temperature for 6 hr. If there follows an interaction between the ERE and GAL4 ER fusion protein, the colony color will turn to blue. The assays of the colony growth were repeated at least three times.

Quantitative liquid β-galactosidase activity assay was performed according to the Yeast Protocols Handbook (CLONTECH Laboratory Inc.). Briefly, transformants (100 µl of stocked yeast) were cultured in a SD lacking His, Leu and Ura with 30-mM 3-AT grown overnight at 30°C with the addition of various amounts of chemicals to the medium. On the following day, 2 ml of the overnight culture were transferred to 8 ml of YPD medium and cultured for 3 hr at 30°C. One hundred fifty µl of culture fluid was centrifuged, and the cells were washed with Z buffer (0.1 M sodium phosphate pH7.0, 10 mM KCl, 1 mM $MgSO_4$) (CLONTECH Laboratory Inc.). The cells were resuspended with 300 µl of Z buffer and used for the β-galactosidase assay using o-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. Activity was normalized to the growth of yeast (OD 600) and assay time.

Examined estrogen and synthetic chemicals

β-estradiol, estrone, estriol, β-estradiol sulfate, estrone sulfate, 2-hydroxyestradiol, 4-hydroxyestradiol and 2-methoxyestradiol (Sigma) were examined transactivity using the screening system. The following synthetic chemicals were examined: dicyclohexyl phathalate, di-*n*-pentyl phthalate, *p*-*n*-pentylphenol, *p*-*t*-octylphenol, *p*-*n*-octylphenol, *p*-*n*-nonylphenol, benzophenone, bisphenol A, *n*-butylbenzene and 1, 3-diphenylpropane (Wako Junyaku, Japan).

RESULTS

Sensitivity of the screening system to β-estradiol

To examine the sensitivity of the established yeast strain to β-estradiol, we performed a β-galactosidase liquid culture. One hundred µl of stocked yeast were cultured with the selection medium SD/-His/-Leu/-U+30mM 3-AT with the addition of various amounts of β-estradiol to the medium. The β-galactosidase activity of yeast was then assayed according to the Material and Methods. The smallest detectable concentration of the β-estradiol was 10^{-11} M in the culture media. At 10^{-9} M β-estradiol in the medium the activity of β-galactosidase rapidly in-

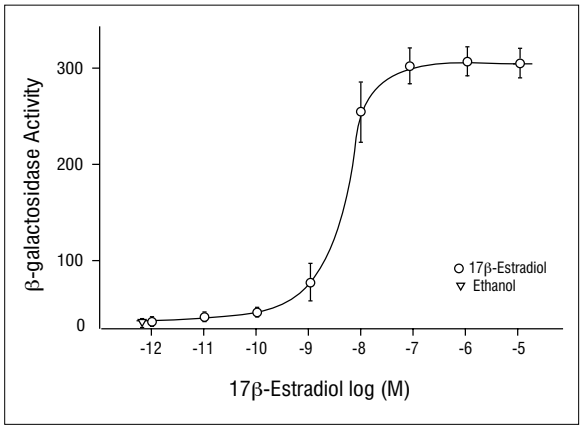

Figure 1. A dose response curve of this endocrine disruptor screening system using β-estradiol. Yeast integrated with the reporter genes were cultured on the SD/-Leu/-U/-His+30 mM 3-AT selection medium with the addition of various amounts of β-estradiol in the liquid culture. β-galactosidase activity was measured according to the Material and Methods. X axis, log M of β-estradiol in the medium; Y axis, β-galactosidase activity. β-galactosidase units of activity were defined as the amount, which was hydrolyzed 1 µmol of ONPG to o-nitrophenol and D-galactose per min. Results are expressed as means ± S. E. for four replicate experiments.

creased, over 10^{-8} M β-estradiol, the β-galactosidase activity was maximal (Figure 1).

Sensitivity of the established screening plate system to β-estradiol

To examine the sensitivity of the established yeast screening plate system to β-estradiol, we plated the yeast strain on the agar plate containing selection medium SD/-His/-Leu/-U+30mM 3-AT. One hundred µl of various concentration of β-estradiol solution in ethanol were spread onto the center of the plate. The solution was allowed to diffuse into the plate after incubating at 30°C for 30 minutes. The stocked yeast was then spread onto the plate containing β-estradiol and incubated at 30°C for four days. The smallest amount of β-estradiol used was 10^{-12} mol that the yeast colonies were could grow well on. Below this concentration the yeast did not grow and did not form the colonies (Figure 2).

Sensitivity of the established screening system to β-estradiol with β-galactosidase filter assays

To examine the minimum detectable concentration of the β-estradiol on the agar selection medium SD/-His/-Leu/-U+30mM 3-AT we assayed the β-galactosidase activity using the filter assay method. The newly formed yeast colonies were copied to the filter paper by the filter lift up method, and β-galactosidase detection limits were examined by the filter assay. The minimum amount of β-estradiol for the appearance of yeast colonies was 10^{-12} mol as examined by the β-galactosidase activity using the filter assay method. The colonies copied to the filter paper turned from white to blue. The detection limit of the β-galactosidase assay was the same as the detection limits of the expression of *HIS* in the selection agar plate. *HIS3* and *lacZ* reporter methods showed similar levels of sensitivity to the concentration of β-galactosidase in the agar plate (Figure 3).

Relative estrogenic transactivity of other non β-estradiol estrogen and estrogen metabolites

The expression of *HIS3* and *lacZ* were dependent on amount of the β-estradiol applied to the screening system. To examine other estrogens in our screening system, we tested estrogen-like compounds including estrogen sulfate and estrogen metabolites. Eight estrogens associated compounds including: estrone, estriol, estrone 3-sulfate, β-estradiol 3-sulfate, 2-hydroxyestradiol, 4-

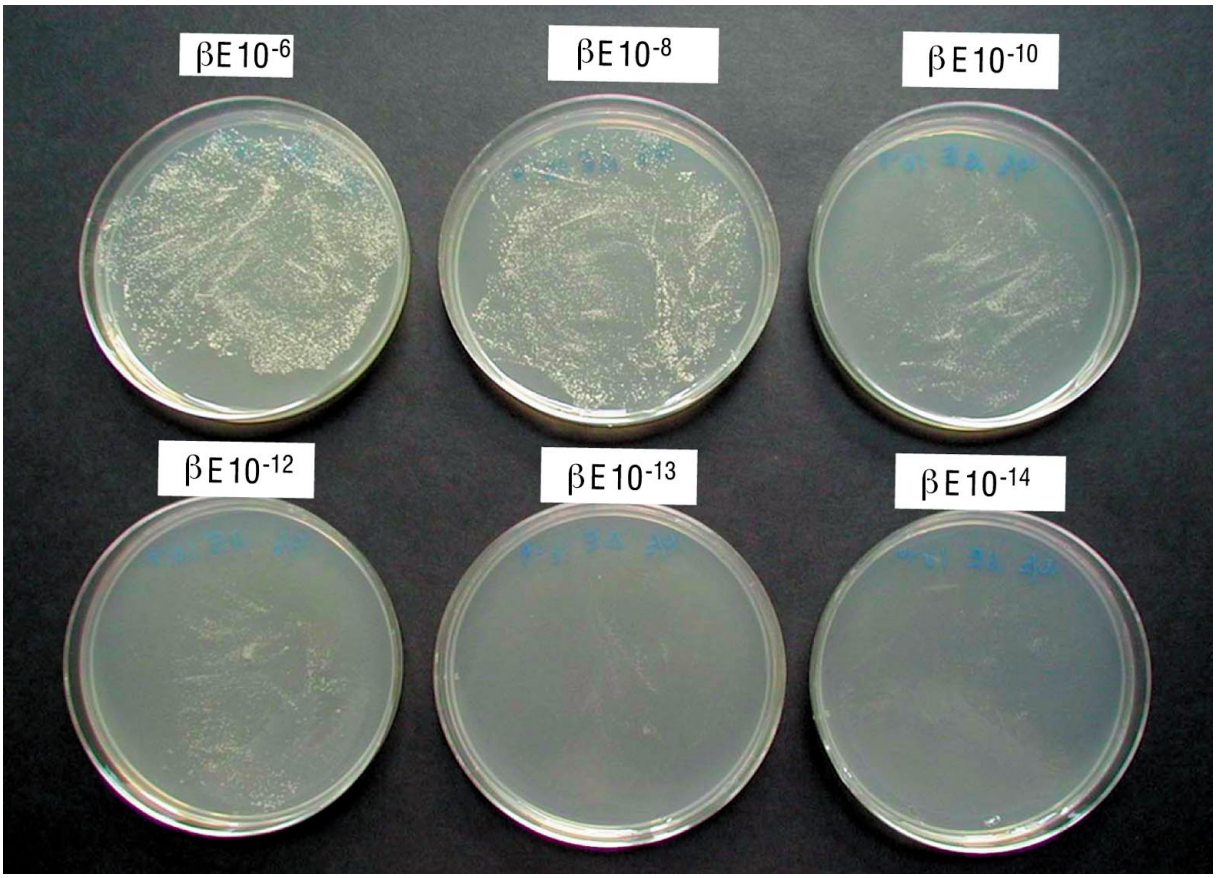

Figure 2. The detection of the β-estradiol with the plate screening system. Yeast integrated with a reporter gene was plated onto an 1.8% agar plate containing SD/-His/-Leu/-Ura+30mM 3-AT medium. After the plates were air-dried, β-estradiol was diluted with ethanol and applied on the plate. The culture plates were incubate at the 30°C for four days and the appearance of the yeast colonies was examined. When the size of the yeast colony on the plate was over 2mm, then the estrogen effect was judged as positive. The values mean the concentration of β-estradiol in the medium (M). (βE, β-estradiol)

Table 1. Relative transactivity of estrogens and their metabolites.

Conc.(log) M/Chemical	β-estradiol	Estrone	Estrone 3-sulfate	Estriol	β-estradiol 3-sulfate	2-hydroxy-estradiol	4-hydroxy-estradiol	2-methoxy-estradiol
–5	+	+	+	+	+	+	+	+
–6	+	+	–	+	+	+	+	+
–7	+	+	–	+	+	+	+	+
–8	+	+	–	+	–	+	+	–
–9	+	+	–	–	–	–	–	–
–10	+	–	–	–	–	–	–	–
–11	+	–	–	–	–	–	–	–
–12	+	–	–	–	–	–	–	–
–13	–	–	–	–	–	–	–	–
–14	–	–	–	–	–	–	–	–
–15	–	–	–	–	–	–	–	–
Ethanol	–	–	–	–	–	–	–	–

Each compound was diluted in ethanol and applied onto the plate. The culture plates were incubated at the 30°C for four days on an agar plate containing SD/-His/-Leu/-U+30mM 3-AT medium. When the size of the yeast colony on the plate was over 2mm, the estrogen effect was judged as positive.
+ The size of colony is over 2mm; – The size of colony is under 2mm

Table 2. Estrogen-like transactivity of synthetic chemicals.

Conc.(log) M/Chemical	β-estradiol	Dicyclohexyl phathalate	Di-n-pentyl phathalate	p-n-Pentylphenol	p-t-Octylphenol	p-n-Octylphenol	p-n-Nonylphenol	Benzophe-none	Bisphenol A	n-Butylben-zene
–5	+	–	–	–	–	–	ND	–	+	ND
–6	+	–	–	+	–	–	–	–	+	–
–7	+	+	–	+	+	+	+	–	–	–
–8	+	+	–	–	+	+	–	–	–	–
–9	+	–	+	–	–	+	–	+	–	–
–10	+	–	–	–	–	_	–	+	–	–
–11	+	–	–	–	–	_	–	–	–	–
–12	+	–	–	–	–	_	ND	–	–	–
–13	–	ND	ND	–	–	ND	ND	–	–	+
–14	–	ND	ND	–	–	ND	ND	–	–	–
–15	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
–16	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ethanol	–	–	–	–	–	–	–	–	–	–

Each chemicals were diluted in ethanol and applied onto the SD/-His/-Leu/-U+30mM 3-AT medium agar plate. The detection of estrogen-like activity was detected by the colony-lift β-galactosidase filter assay. Colonies that turned blue produce β-galactosidase after transactivation of the lacZ reporter.
The turned blue colony was judged as positive (+). The olony did not turn blue was judge as negative (–); ND, Not determined

hydroxyestradiol and 2-methoxyestradiol were tested. The transactivity was examined by the expression of both *HIS3* and *lacZ* with various kind of estrogen diluted by ethanol (Table 1). The activity of β-estradiol is 1000 times that of estrone, 10,000 times that of estriol and 100,000 times of β-estradiol 3- sulfate. The transactivity of metabolites of β-estradiol were 1/10,000 times before metabolite.

The estrogenic activity of synthetic chemicals

We examined whether the screening systems are able work to detect the estrogen-like activity of synthetic chemicals. Nine chemicals which are thought have endocrine-disrupting ability were dissolved in ethanol and prepared at various concentration. All nine chemicals were studied in our screening system. Using this method, chemicals were shown to have estrogen-like activity (Table 2). The estrogen activity of the synthetic chemicals did not show any dose dependent transactivity. Benzophenone had estrogen activity at between 10^{-9} and 10^{-10} mol. However, under a concentration of 10^{-8} mol or over a concentration of 10^{-11} mol benzophenone did not display any transactivity.

The analysis of estrogen-like activity

The estrogen effect was examined using the liquid culture method in order to assess whether the estrogen-like effect of the synthetic chemicals was characteristic for plate method. The yeast was cultured in liquid culture medium containing SD/-His/-Leu/-U+30mM 3-AT at 30°C for two days and the β-galactosidase assayed as previously mentioned. Between the concentration of 10^{-8} M and 10^{-10} M benzophenone yeast would grow. Benzophenone, therefore has a narrow range activity. However, the β-galactosidase activity was increased in a dose dependent manner with *p-t*-octylphenol, at a concentration of 10^{-7} M. The β-galactosidase activity was also increased in a dose

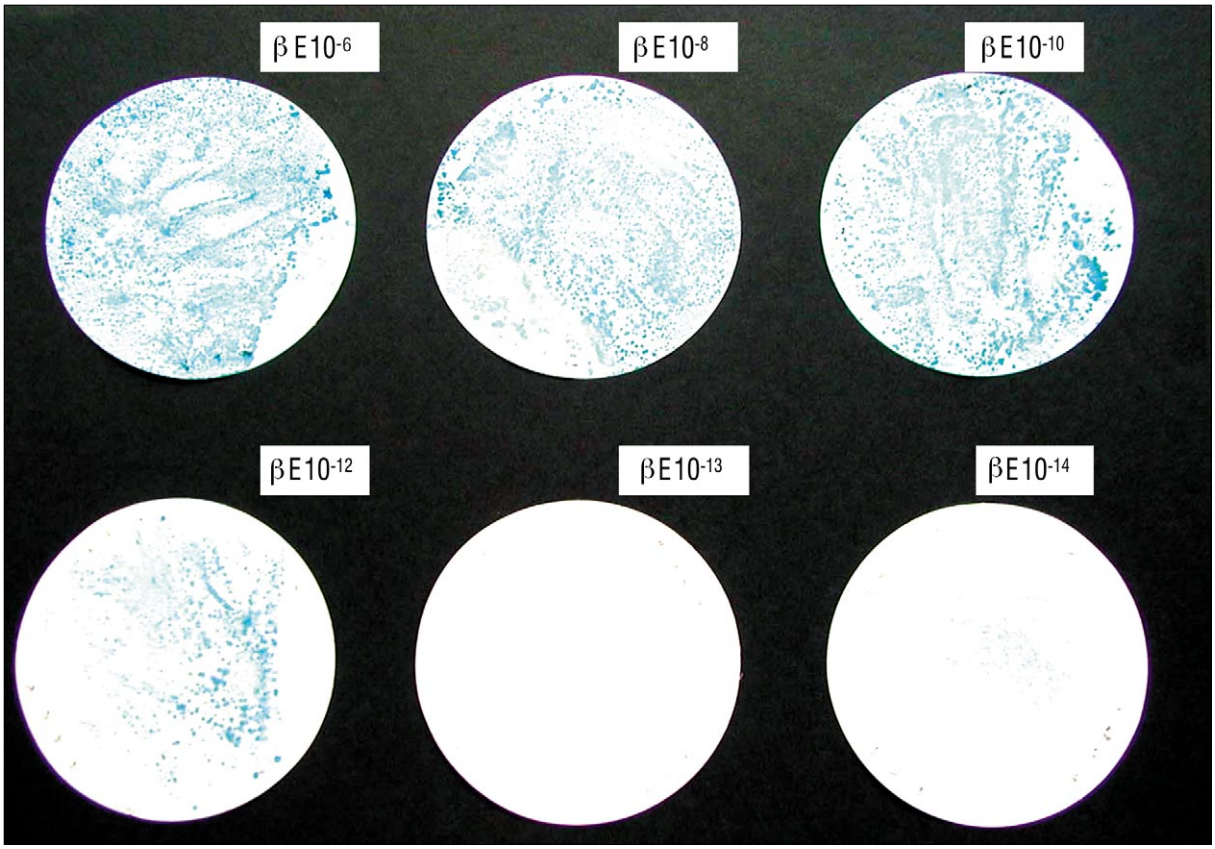

Figure 3. The colony-lift β-galactosidase filter assay to detect the β-estradiol transactivity. Yeast was incubated on the agar plate containing SD/-His/-Leu/-U+30mM 3-AT medium. Various concentration of β-estradiol diluted in ethanol was applied on to the plate. The culture plates were incubated at the 30°C for four days and the colonies were lifted on to the filter paper. The filter papers were soaked with X-Gal solution and incubated at room temperature. Colonies turned blue if they produced β-galactosidase after transcription activation of the *lacZ* reporter gene. The figures refer to the mean values for the concentration of β-estradiol applied on the agar plate. (βE, β-estradiol)

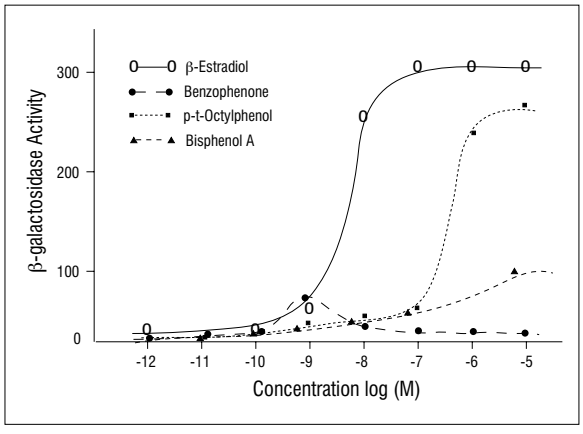

Figure 4. Analysis of estrogen-like activity of synthetic chemicals. Yeast was cultured on the SD/-Leu/-U/-His+30 mM 3-AT selection medium with the addition of various amounts of chemicals in the liquid culture. β-galactosidase was assayed after the addition of benzophenone (■), p-t-octylphenol (▲) and bisphenol A (●). X axis, log M of chemicals in the medium; Y axis, β-galactosidase activity. These experiments were repeated three times with similar results.

dependent manner with bisphenol A at a concentration of 10^{-8} M (Figure 4).

Discussion

Endocrine disruptors are chemical substances, which inhibit the normal function of the endocrine system. Their mechanism of action is not well understood. There are many chemicals produced every day, which have endocrine disrupting ability. In this study, we have developed an easy and low cost screening system and adapted this screening system to detect endocrine disruptors. Endocrine disruptors are closely related to molecules involved in the endocrine reproductive system, and this disturbance of the endocrine system is known to have a significant effect on humans. We are therefore, first to have developed a detection system for estrogen or estrogen-like chemicals. Estrogen passes through the plasma membrane and binds to nuclear estrogen receptor that subsequently affects gene expression via a second messenger or signal transduction pathway. This screening system utilizes the features of nuclear receptors to detect DNA protein interactions with a ligand. We applied this One-hybrid System to an *in vivo* assay system using yeast to develop the screening system.

Recently the estrogen receptor unlike conventional receptors such as ERβ has been reported that its expression pattern differs from that of ERα expression [14–16]. The ERβ has a similar physiological effect to ERα. This screening assay was made using the ERα cDNA. In the liquid culture, the yeast sensitivity to detect estrogen is 1×10^{-11} M β-estrogen. The estrogen sensitivity is also dose-dependent. The minimum detectable estrogen concentration is approximately equal to the concentration of estrogen in human serum, and it is possible to quantitatively measure the estrogen in a given organism. The sensitivity of our screening system seems to have the same sensitivity as the previous described the yeast based estrogen assay [17,18] or the luciferase reporter assay in mammalian cell [19,20]. The screening method used a plate, yeast to form a colony and β-galactosidase expression. The minimum detectable of β-estrogen was 1×10^{-12} mol in the plate selection medium. In the liquid culture we have to assay β-galactosidase after yeast culture. The culture-assay method is more time consuming and complicated, but the plate screening method is relatively simple and easier than the previously described YES system [21,22]. We can examine whether chemicals have estrogen activity and detect the transactivity from the growth of macroscopic yeast colonies.

Although this is an assay system to detect the β-estradiol, we explored whether this screening system can be applied to other estrogens, sulfated steroids and estrogen metabolites. Examination using the established screening system, revealed that the transactivity of estrone is 1/1000 times that of β-estradiol and the transactivity of estriol is 1/10,000 times that of β-estradiol. The sulfated estrogen, which has the sulfate group in the 3-β position in the sterol structure; estrone 3-β sulfate and β-estradiol sulfate have 1/100,000 times activity of β-estradiol effect. We show that the sulfuric acid estrogen does not manifest any estrogen action, as it does not bind to ER [23,24]. The estrogen effect of 2-hydroxyestradiol and 4-hydroxyestradiol, which is an estrogen metabolite, was 1/10,000 times less than the estrogen transactivity, in addition, the estrogen effect was abated, after it was metabolized [25,26]. Nine chemicals, which have an endocrine disrupting effect, were examined for estrogen transactivity with the plate screening system. Though nine chemicals showed some estrogen-like effects, they did not show dose dependent estrogenic-transactivity. The endocrine disturbing action of synthetic chemicals was not manifest in a dose dependent manner and that was a characteristic of synthetic compounds. It is estimated that this demonstrated at a specific concentration of the synthetic chemical with an endocrine disrupting action [27].

CONCLUSIONS

Estrogens bind to not only estrogen receptor but also androgen. Estrogen affect spermatogenesis and it is supposed that estrogen-like chemical may bind to the androgen receptor. Estrogen disruptors can affect downstream signal transduction to alter spermatozoon numbers [28]. Although the endocrine screening systems are for detection of the estrogen-like action of endocrine disruptors, an androgen screening system also important to examine the effect on the male endocrine system. It seems that an androgen receptor chemicals screening system, which is similar to the screening system for estrogen-like chemicals must be developed. Only then synthetic chemicals will be safely assessed in the future.

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