

## Assessment of estrogenic activity of natural compounds using improved E-screen assay

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**KEY WORDS** estrogen receptors; antisense RNA; estrogens

### ABSTRACT

**AIM:** To improve E-screen assay and make it more accurate to screen estrogenic compounds. **METHODS:** Estrogen receptor antisense RNA expression plasmid (pCASER) was constructed and introduced into MCF-7 with lipofectAMINE<sup>TM</sup>, and positive clones were screened out with G418. PCR amplification was employed to identify whether estrogen receptor (ER) cDNA fragment had been inserted into MCF-7 cell genomes. Western blot was applied to detect the expression of ER. Cell growth was determined by MTT assay. **RESULTS:** One ER antisense clone (MTASER) had been screened out. The effects of 17 $\beta$ -estradiol, genistein, droloxifen, miyabenol C, and kobophenol A on MCF-7 were stronger than those effects on MTASER. Epidermal growth factor (EGF) had equivalent stimulatory effects on the proliferation of MCF-7 and MTASER. **CONCLUSION:** The improved E-screen assay could screen estrogenic compounds more accurately than original E-screen assay did.

### INTRODUCTION

A series of *in vitro* assays have been developed for detection of potential estrogens. One of the most classical assays is cell proliferation assay (E-screen assay)<sup>[1]</sup>. In this *in vitro* test system, the ability of a substance to stimulate the growth of estrogen sensitive cell lines like MCF-7 or T-47D is measured<sup>[2]</sup>. Such estrogen enhancement may arise from the estrogen receptor-mediated process<sup>[3]</sup>. But many chemicals that

have no estrogenic effects such as cytokines, growth factors, mitogens, and nutrients do stimulate MCF-7 cell line through no-estrogen receptor-mediated process. So the most disadvantage of this method is that a considerable number of substance gives positive results in the E-screen without exerting estrogenic activity. Now, we constructed estrogen receptor antisense RNA expression plasmid and introduced it into MCF-7, and screened out positive clone (MTASER) in which the expression of estrogen receptor (ER) was decreased. Using not only MCF-7 but also MTASER in E-screen assay, we could detect how much the compound depended on estrogen receptor to stimulate MCF-7.

17 $\beta$ -Estradiol (E<sub>2</sub>) and genistein (Gen) are the most classical endogenous estrogen and phytoestrogen respectively<sup>[4,5]</sup>. Droloxifen (Dro) at low concentration also show estrogenic activity<sup>[6]</sup>. Miyabenol C (Miy C) and kobophenol A (Kob) were extracted from *Caragana Sinica*<sup>[7]</sup>. We have confirmed that Miy C and Kob A have estrogenic activity, and they could compete with 17 $\beta$ -estradiol binding to estrogen receptor, promote estrogen receptor-mediated transcription, and stimulate the growth of MCF-7 and osteoblast (unpublished). Epidermal growth factor (EGF) is one kind of growth factors. So we examined the effect of E<sub>2</sub>, Gen, Dro, Kob A, Miy C, and EGF on the growth of MCF-7 and MTASER, and compared the different effects of these compounds on the two cell lines in this paper.

### MATERIALS AND METHODS

**Drugs and reagents** E<sub>2</sub>, Gen, EGF, and anti-ER antibody (IgG of rabbit antiserum) were purchased from Sigma. Dro was a gift of Prof XIA Peng (Institute of Pharmacy, Fudan University). Miy C, Kob A, Genestein, Dro, and E<sub>2</sub> were dissolved in Me<sub>2</sub>SO with stock concentration of 0.1 mol/L. LipofectAMINE<sup>TM</sup> reagent was purchased from Gibco BRL. MCF-7 cells that expressed ER was obtained from ATCC. pSG5-ER

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which contains a human ER cDNA was a gift of Prof Stefan NILSSON (KAB BIO AB, Sweden).

**Cell culture** All cells were cultured in RPMI-1640 supplemented with 10 % calf serum, 10 mg/M bovine insulin, penicillin 100 mg/L, streptomycin 100 mg/L, L-glutamine 2 mmol/L at 37 °C, under a humidified atmosphere of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. Prior to the beginning of each experiment, all cells were grown in estrogen-free media for at last 2 d. Estrogen-free media substituted phenol red-free RPMI-1640 and 10 % heat-inactivated 3 × charcoal-stripped calf serum<sup>[8]</sup>.

**Plasmid** pCASER was constructed by inserting 1.8 kb *EcoR* I and *Bam* H I -digested wild-type ER cDNA fragment which was isolated from plasmid pSG5-ER into the *Bam* H I and *EcoR* I site of vector pCDNA3.

**Transfection** MCF-7 cells were transfected with pCASER using LipofectAMINE™ reagent, and neomycin-resistant clones were identified and characterized.

**Oligonucleotide primers** Oligonucleotides, 5'-A-TACGAAASGACCGAAGAGGAG-3' and 5'-CCAGACGAGACCAATCATCA-3' complementary to regions located at exon 4 and exon 5 of ER were used as primers for PCR amplification<sup>[9]</sup>.

**Genome DNA extraction** The genome DNA was extracted via modified Gross-Bellard's method<sup>[10]</sup>.

**PCR amplification** PCR was performed in 50 μL reaction mixture containing 10 pmol of each primer, *Taq* DNA polymerase 1.5 U, dNTP 400 μmol/L, and 1 × *Taq* reaction buffer. The temperature profile was as follows; 1 cycle of denaturation at 94 °C; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 1 cycle of extension at 72 °C for 5 min. PCR products were analyzed by 1 % agarose gel electrophoresis and stained with ethidium bromide.

**Western blot** According to the method<sup>[11]</sup>, Whole-cell extracts were prepared by direct lysis of PBS washed cells in a lysis buffer (2 % SDS, 10 % glycerol, Tris-HCl 0.15 mol/L), followed by immersion in boiling water bath for 5 - 10 min. Equal amounts of protein were added to each lane of 10 % polyacrylamide gel with 3 % stacking gel.

**Growth assays** Cells were seeded into each well of 96-well plate (2 × 10<sup>3</sup> cells/well) in 100 μL of estrogen-free media on d 0. The following day (d 1), this media was removed, and 200 μL of media containing the appropriate compound was added. All compounds were dissolved in Me<sub>2</sub>SO and added to media at a 1:1000 dilution. Media was changed on d 5, and experiments

were ended on d 8. The cell growth was determined by MTT assays. All experiments were performed in triplicate<sup>[12]</sup>.

**Statistical analysis** Results were expressed as  $\bar{x} \pm s$  and compared by *t*-test.

## RESULTS

### Construction and characterization of pCASER

After digestion of pCASER with *Sal* I, three DNA fragments of about 0.9, 2.2, and 4.1 kb in length were occurrence (Fig 1a), and digested with *Bgl* II, two DNA fragments of about 1.2 and 6.0 kb could be obtained (Fig 1b). The results indicated that pCASER containing ER cDNA fragment placed in an antisense orientation had been constructed.

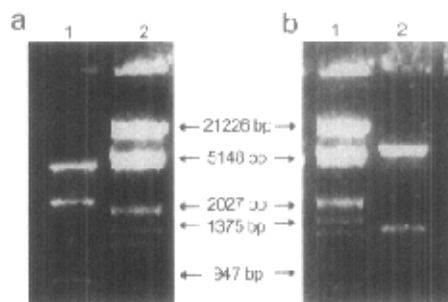


Fig 1. Restriction analysis of pCASER. (a) 1: pCASER/*Sal* I; 2: λDNA/*Hind* III + *EcoR* I; (b) 1: λDNA/*Hind* III + *EcoR* I; 2: pCASER/*Bgl* II.

**Characterization of MTASER** PCR analysis of MTASER genome DNA using ER cDNA specific primers, one DNA fragment about 400 bp in length was occurrence. The results showed that ER cDNA fragment had been inserted into MCF-7 cell genomes (Fig 2).

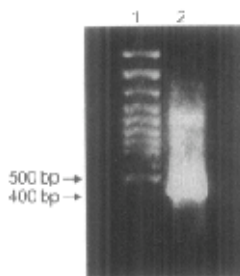


Fig 2. PCR analysis of MTASER genome DNA. 1) 100 bp DNA ladder; 2) MTASER genome DNA.

Western blot was applied to detect the expression of ER. The level of ER in MTASER cell clone was about as high as 1/12 that in MCF-7 cell (Fig 3).

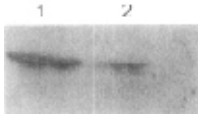


Fig 3. Down-regulation of ER in antisense clone (MTASER); the same amount of protein was applied for Western blot; 1) whole-cell extract protein from MCF-7; 2) whole-cell extract protein from MTASER.

**Effect of E<sub>2</sub> and Dro on the growth of MCF-7 and MTASER** E<sub>2</sub> at concentration between 1 pmol/L and 100 nmol/L significantly stimulated the growth of MCF-7. Only at concentration 1 pmol/L and 10 nmol/L E<sub>2</sub> could significantly stimulate the growth of MTASER. The effects of E<sub>2</sub> on MCF-7 were stronger than those effects on MTASER (Fig 4A).

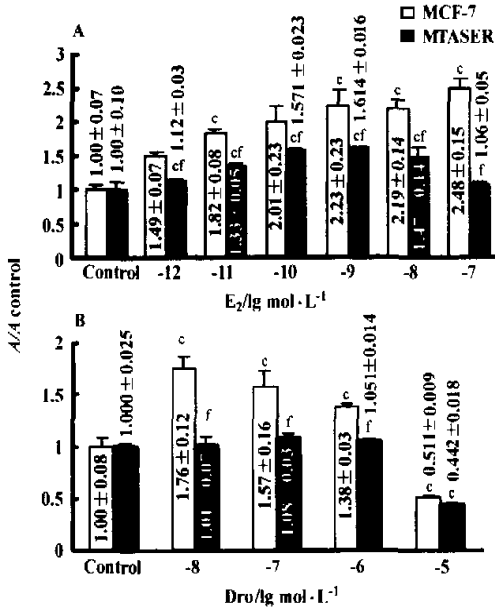


Fig 4. Effect of E<sub>2</sub> (A) and Dro (B) on the growth of MCF-7 and MTASER. n = 3 experiments (each done in 3 wells).  $\bar{x} \pm s$ . <sup>c</sup>P < 0.01 vs control. <sup>f</sup>P < 0.01 vs the same dose of compound on MCF-7.

Dro at concentrations of 10, 100, and 1000 nmol/L significantly stimulated the growth of MCF-7, but did not stimulate the growth of MTASER at the same concentrations (Fig 4B).

**Effect of Miy C and Kob A on the growth of**

**MCF-7 and MTASER** Both Miy C and Kob A had stimulatory effects on the proliferation of MCF-7. At concentration of 100 nmol/L of Miy C and Kob A could slightly stimulated the growth of MCF-7. The maximum stimulation of Miy C and Kob A on cell growth occurred at concentrations of 10 μmol/L and 100 μmol/L respectively. Only at concentration of 10 μmol/L, Miy C and Kob A begun to stimulation the growth of MTASER. The effects of Miy C and Kob A on MCF-7 were stronger than those on MTASER (Fig 5).

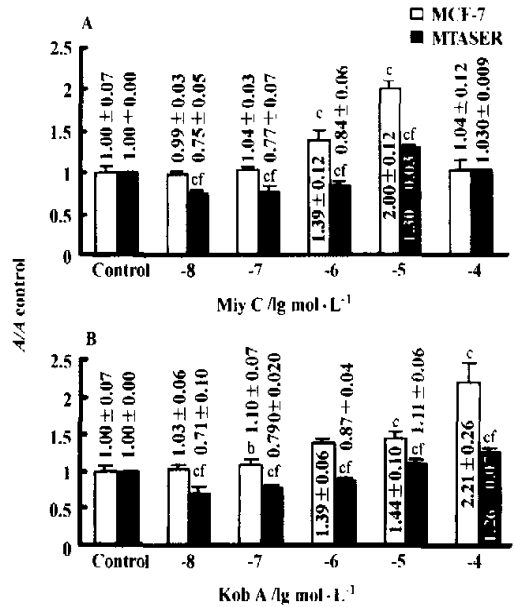


Fig 5. Effect of Miy C (A) and Kob A (B) on the growth of MCF-7 and MTASER. n = 3 experiments (each done in 3 wells).  $\bar{x} \pm s$ . <sup>b</sup>P < 0.05, <sup>c</sup>P < 0.01 vs control. <sup>f</sup>P < 0.01 vs the same dose of compound on MCF-7.

**Effect of Gen on the growth of MCF-7 and MTASER** At concentration between 1 nmol/L and 1 μmol/L Gen significantly stimulated the growth of MCF-7. Gen slightly stimulated the growth of MTASER at concentration between 1 nmol/L and 1 μmol/L (Fig 6).

**Effect of EGF on the growth of MCF-7 and MTASER** EGF had equivalent stimulatory effects on the proliferation of MCF-7 and MTASER at all the concentrations tested (Fig 7).

**DISCUSSION**

Estrogens have been shown to exert a variety of

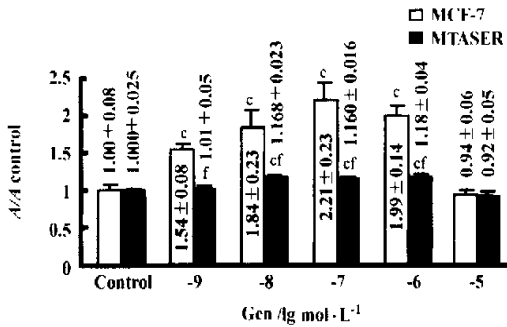


Fig 6. Effect of Gen on the growth of MCF-7 and MTASER.  $n = 3$  experiments (each done in 3 wells).  $\bar{x} \pm s$ .  $^c P < 0.01$  vs control.  $^c P < 0.01$  vs the same dose of compound on MCF-7.

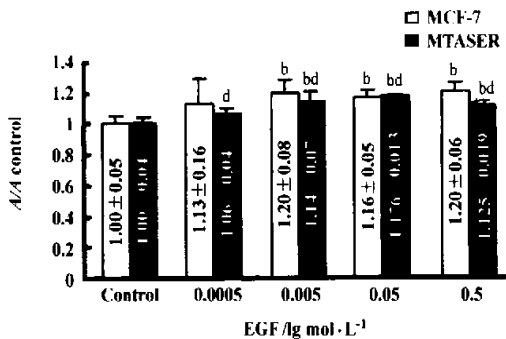


Fig 7. Effect of EGF on the growth of MCF-7 and MTASER.  $n = 3$  experiments (each done in 3 wells).  $\bar{x} \pm s$ .  $^b P < 0.05$  vs control.  $^d P > 0.05$  vs the same dose of compound on MCF-7.

beneficial effects on men and women. It is recognized to provide protection against osteoporosis, heart attack, and other cardiovascular problems, and possibly Alzheimer's disease. Recently, mounting evidence suggests that plant-derived estrogens (phytoestrogens) such as genistein, may exert beneficial effects on the above-mentioned chronic diseases. Phytoestrogens have not only estrogenic activity but also some anti-tumor and anti-oxidized activity. For example, genistein is a specific inhibitor of tyrosine-specific protein kinase<sup>[13,14]</sup> and Miy C can inhibit protein kinase C<sup>[15]</sup> at high concentration, and then they can inhibit the growth of cells.

Antisense-based inhibition of gene expression is mainly through RNA-RNA duplex formation and subsequent degradation of the double-strand transcript<sup>[16]</sup>. Down-regulation of gene expression using antisense RNA can be approached in two ways one of which is directly

introducing synthetic antisense oligonucleotide into cells, another is constructing antisense RNA expression plasmid and introducing it into cells. To decrease the expression of ER, ER antisense RNA expression plasmid using pCDNA3 as expression vector was constructed and introduced into MCF-7, and we screened out positive clone MTASER. In MTASER, ER cDNA fragment placed in an antisense orientation has been stably integrated with MCF-7 genome DNA, and the expression of ER was decreased by about 92 %.

The compounds that have estrogenic activity such as E<sub>2</sub>-, the classical endogenous estrogen, Gen-phytoestrogen, Dro- which exhibits estrogenic activity only at low concentration and antiestrogenic activity at high concentration, Miy C and Kob A-phytoestrogens, two new kinds of phytoestrogens, all could significantly stimulate the growth of MCF-7 at some appropriate concentration. At the same time, these compounds have no stimulatory effects or have weak stimulatory effects on MTASER. The compounds that have no estrogenic activity such as EGF would have similar stimulatory effects on the two cell lines. Comparing the difference between the effects on MCF-7 and MTASER, we could distinguish the compounds that have no estrogenic activity but can stimulate the growth of MCF-7 from estrogenic substance just only through using E-screen assay.

The advantage of E-screen is easy to perform and cheap, but the accuracy is lower than other test systems as the receptor binding assay, report gene assay, etc<sup>[17,18]</sup>. When the effects of natural compounds on MCF-7 and MTASER combine to be examined, the accuracy of E-screen has been improved, and the advantage has been also reserved.

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应用改良的 E-screen 实验评估天然物质的类雌激素活性

R96 A

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关键词 雌激素受体; 反义 RNA; 雌激素类

目的: 改良 E-screen 实验, 提高其筛选具有雌激素样作用物质的准确性. 方法: 构建雌激素受体反义 RNA 表达质粒 pCASER, 以脂质体转染 MCF-7 细胞, G418 筛选阳性克隆. PCR 检测雌激素受体的 DNA 是否整合于 MCF-7 细胞的基因组 DNA 中, Western blot 检测雌激素受体的表达; MTT 法检测细胞的增殖. 结果: 筛选出一株雌激素受体反义 RNA 表达克隆 (MTASER). 17 $\beta$ -雌二醇, 金雀异黄素, Droloxifen, Miyabenol C 和 Kobophenol A 在一定浓度均可促进 MCF-7 细胞增殖, 且对 MCF-7 的促增殖作用大于对 MTASER 的促增殖作用; 表皮生长因子对两株细胞的促增殖作用的差别无显著意义. 结论: 改良的 E-screen 实验筛选具有雌激素样作用物质的准确性高于通常的 E-screen 实验.

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