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A Cell-based High-throughput Screening Assay for Farnesoid X Receptor Agonists[1]

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Objective To develop a high-throughput screening assay for Farnesoid X receptor (FXR) agonists based on mammalian one-hybrid system (a chimera receptor gene system) for the purpose of identifying new lead compounds for dyslipidaemia drug from the chemical library. **Methods** cDNA encoding the human FXR ligand binding domain (LBD) was amplified by RT-PCR from a human liver total mRNA and fused to the DNA binding domain (DBD) of yeast GAL4 of pBIND to construct a GAL4-FXR (LBD) chimera expression plasmid. Five copies of the GAL4 DNA binding site were synthesized and inserted into upstream of the SV40 promoter of pGL3-promoter vector to construct a reporter plasmid pG5-SV40 Luc. The assay was developed by transient co-transfection with pG5-SV40 Luc reporter plasmid and pBIND-FXR-LBD (189-472) chimera expression plasmid. **Results** After optimization, CDCA, a FXR natural agonist, could induce expression of the luciferase gene in a dose-dependent manner, and had a signal/noise ratio of 10 and Z' factor value of 0.65. **Conclusion** A stable and sensitive cell-based high-throughput screening model can be used in high-throughput screening for FXR agonists from the synthetic and natural compound library.

Key words: Farnesoid X receptor; Agonist; High-throughput screening; Chimera

INTRODUCTION

Nuclear hormone receptors constitute a superfamily of ligand activating transcription factors that play a critical role in human development and adult physiology. Farnesoid X receptor (FXR), a member of the nuclear hormone receptor superfamily, is primarily expressed in the liver, kidney, and intestine[1]. FXR functions as a heterodimer with the 9-*cis*-retinoic acid receptor (RXR) and binds to farnesoid X receptor response elements (FXREs), a specific DNA sequence comprising two inverted hexamer repeats separated by one nucleotide (IR-1) in the target promoter or bile acid response elements found in the promoter of FXR-responsive genes[1-2]. FXR regulates the expression of various transport proteins and biosynthetic enzymes which are crucial to the physiological maintenance of lipids, cholesterol and bile acid homeostasis[3].

FXR agonists have demonstrated a critical role in maintaining cholesterol and bile acid homeostasis[4], and FXR has become a valuable target for the identification of novel drugs to treat dyslipidaemia and cholestasis. Regulation of FXR through small-molecule drugs represents a promising therapy for diseases resulting from lipid, cholesterol and bile acid abnormalities[5-8].

Mammalian one-hybrid system, also known as Chimera receptor gene system, represents an extremely powerful method which has been developed for detecting ligands/nuclear receptor interactions *in vitro*[9-10]. The one-hybrid system is based on co-transfection mammalian cells with a reporter plasmid containing a yeast GAL4 DNA binding site and a GAL4-chimera fusion express plasmid. The transactivity of ligands to nuclear receptors is evaluated by monitoring expression of

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the luciferase gene induced by ligands.

To screen new FXR agonists from synthetic and natural compounds library, we developed a cell-based high-throughput screening model based on the chimera receptor gene system. The expression of plasmid confused hFXR-LBD (amino acids 189-472) to GAL4 (1-147). Reporter plasmid was constructed by inserting five copies of GAL4 DNA binding site into the upstream of SV40 promoter, a strong promoter of mammalian cells. After optimization, the high signal/noise ratio and acceptable *Z'* factor proved that the assay was quantitative, stable and sensitive.

In this paper, we present the details of plasmid construction, optimization of the cotransfection condition and validation of the FXR agonist screening model.

MATERIALS AND METHODS

Reagents and Materials

Chenodeoxycholic acid (CDCA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Human hepatocytes (L02 cell line) were obtained from China Center for Type Culture Collection (CCTCC). Oligonucleotide was synthesized by Shanghai Sangon Co. Ltd. (Shanghai, China).

Plasmids

cDNA encoding human FXR-LBD (amino acids 189-472) was amplified by RT-PCR from a human liver total mRNA (Clontech) according to methods described by ThermoScript™ RT-PCR System (invitrogen). Briefly, the forward primer (5'-actgg atccgtatgggaatgttggctgaatg-3') and the reverse primer (5'-Atcggtacctcactgcacgtcccagatt-3') were used to amplify human FXR-LBD with the following regime: 30 cycles with denaturing at 94℃ for 30 s, annealing at 58℃ for 1 min, and extension at 72℃ for 1 min followed by a final extension at 72℃ for 10 min. The product of PCR was then cloned into pGEM-T vector and sequenced. Chimera expression plasmid pBIND-FXR (LBD) was constructed by fusing the FXR-LBD fragment to 3' of the DNA binding domain of yeast GAL4 of pBIND (Promega), and maintained on an open reading frame. Reporter plasmid pG5-SV40-Luc (firefly luciferase) was constructed by inserting five copies of the GAL4 DNA binding site (CGG AGTACTGTCC T) (synthesized at invitrogen) into upstream of the SV40 promoter of pGL3-promoter vector (Promega). The integrity of all constructs was verified by DNA sequence and restriction enzymatic analysis.

Cell Culture and Transient Transfections

Human hepatocytes (L02 cell line) were grown at 37℃ in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) and antibiotics (streptomycin/penicillin) in a humidified atmosphere containing 5% CO_2. Twenty-four hours before transfection, cells were seeded into a 96-well microplate at a concentration of 5×10^4 cells/well in RPMI-1640 medium supplemented with 10% FBS. Transient co-transfection was then performed using pG5-SV40-Luc and GAL4-FXR chimera expression plasmid pBIND-FXR (LBD) by delivering liposome with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Six hours post transfection, FXR ligands or samples were added. Twenty-four hours after incubation with ligands, cells were harvested in lysis buffer (0.1 mol/L Tris/HCl, pH 6.8, 2 mmol/L EDTA, 0.25% Triton X-100), and cell lysates were analyzed for firefly and Renilla luciferase activity using the dual-luciferase® reporter assay system (Promega Corp. Cat. # E1910). Luciferase activity was measured using a microplate reader (Wallac 1420 Victor2, PerkinElmer). Renilla luciferase expressed by pBIND-FXR (LBD) was used for normalization of the transfection efficiency. All experiments were performed at least three times. Because of the sensitivity of cells to organic solvent, the amount of DMSO was limited to 0.5% of the total liquid volume. The fold induction of luciferase activity was calculated by avg. signal of sample /avg. signal of DMSO.

Assay Performance

In a routine screening process, positive (50 μmol/L CDCA) and negative (0.5% DMSO) controls in quadruplicate were included in each 96-well plate for quality control assessment. The signal/noise ratio (S/N) or the signal window was calculated using avg. max.signal (positive control) /avg. min. signal (DMSO). *Z'* factor was calculated as previously described[10]. Data were expressed as $\bar{x}\pm s$ for triplicate, representing at least three independent experiments.

$$\text{Z' factor}=\frac{(\text{avg.maxim signal}-3\text{SDmax})-(\text{avg.minimum signal}+3\text{SDmin})}{(\text{avg.maxim signal-avg.minimum signal})}$$

RESULTS

Plasmid Construction

A 887 bp fragment coding region 189-472 of human FXR LBD was obtained from total mRNA of human liver by RT-PCR (Fig. 1), and cloned into the pGEM-T easy vector to confirm its sequence. Compared with the published hFXR encoding sequence (GenBank Accession No. NM_005123), the cloned FXR ligand-binding domain nucleotide sequence was found to have three mutations at 606 T→C, 729 A→G, 1314 A→G, respectively, but its encoding amino acid sequence was identical to published sequence. Then the Kpn1/BamH1 fragment of FXR (LBD) was excised and confused to the 3' of GAL4 (DBD) of the pBIND to obtain the chimera expression plasmid pBIND-FXR (LBD) (Fig. 2).

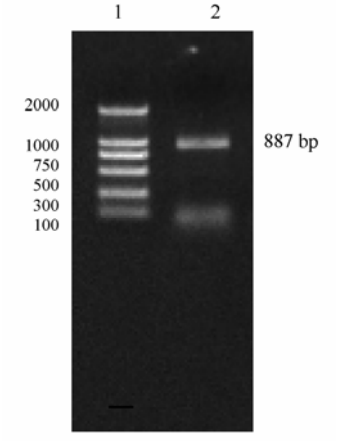

FIG. 1. Analysis of RT-PCR products. Lane 1: DL2000 marker; lane 2: PCR products of FXR-LBD.

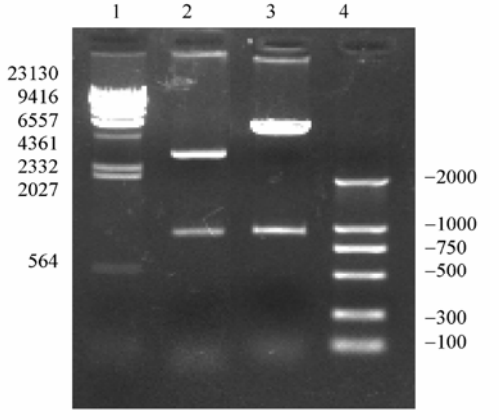

FIG. 2. Restriction enzymatic analysis of recombinant plasmids. Lane 1: λHindIII Marker; lane 2: pGEM-T-FXR (LBD) digested with BamH and KpnI to get 887 bp and 3150 bp fragments; lane 3: pBIND-FXR (LBD) digested with BamH and KpnI to get 887 bp and 6310 bp fragments; lane 4: DL2000 Marker.

Five copies of the yeast GAL4 DNA binding site were synthesized and inserted into upstream of the SV40 promoter of pGL3-promoter vector (Promega) to construct reporter plasmid pG5-SV40-Luc, which was confirmed by restriction enzymatic analysis with Kpn1 and Sma1.

Optimization of Cotransfection Condition

Since many factors would significantly affect the efficiency of cotransfection, to identify optimum conditions of transient cotransfection assays, optimization of the transfection in 96-well plates was performed. The optimum molar ratio of reporter vector to expression vector was first confirmed by cotransfection at 0.2 μg /well of DNA with different molar ratios of the reporter vector to the expression vector of 20:1, 10:1, 5:1, 1:1, 1:5, and 1:10, and 0.5 μL/well of Lipofectamine™ 2000 (Invitrogen). After 6 hours of transfection, the cells were incubated with 50 μmol/L CDCA for about 24 h, and the luciferase activity was measured. The result (Fig. 3) showed that when the molar ratio of the reporter vector to the expression vector was 10:1, the firefly luciferase activity was the highest.

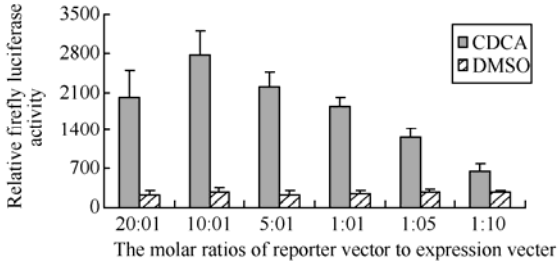

FIG. 3. Optimization of molar ratios of reporter vector to expression vector. Transactivation of 50 μmol/L CDCA was assayed at different molar ratios of reporter to expression vector.

With a fixed molar ratio (10:1) and the volume of Lipofectamine™2000 (0.5 μL /well), cotransfection was performed at the concentration of DNA per well ranging from 0.1 to 0.6 μg. The results (Fig. 4) showed that the luciferase activity value increased with the increasing concentration of DNA. When the concentration of DNA was over 0.5 μg per well, the luciferase activity value decreased. The highest fold induction in luciferase activity (Fig. 5) of 50 μmol/L CDCA could be obtained at 0.3 μg DNA per well.

Validation of Screening Model

To validate the cell-based screening model, transient cotransfection was performed in 96-well plates

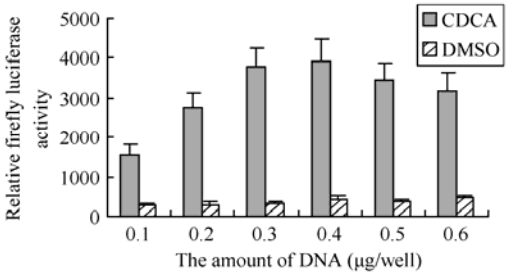

FIG. 4. Optimization of the amount of DNA per well. Transactivation effect of 50 μmol/L CDCA was assayed at different concentrations of DNA per well ranging from 0.1-0.6 μg with the molar ratio (10:1) of reporter vector to expression vector and 0.5 μL/well of lipofectamine™2000.

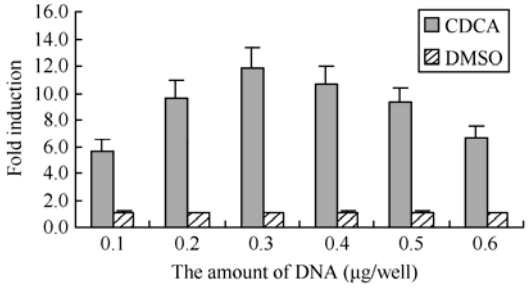

FIG. 5. Fold induction of luciferase activity of 50 μmol/L CDCA at different concentrations of DNA per well ranging from 0.1-0.6 μg with the molar ratio (10:1) of reporter vector to expression vector and 0.5 μL/well of lipofectamine™2000.

at the optimum condition: the molar ratio of reporter plasmid and chimera express plasmid was 10:1, 0.3 μg/well of DNA, 0.5 μL/well of lipofectamine™ 2000. Six hours post transfection, the cells were treated with CDCA diluted from 50 μmol/L to 0.05 μmol/L. The result (Fig. 6) showed that luciferase activity could be induced by CDCA in a dose-dependent manner,

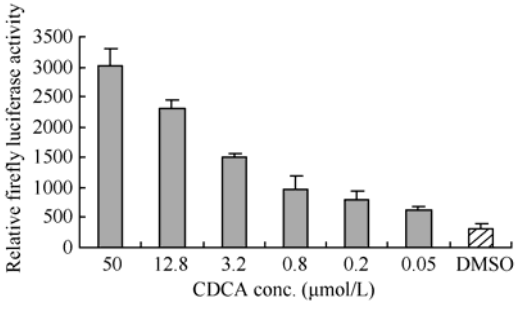

FIG. 6. The effect of CDCA on the induction of firefly luciferase activity. After 6 hours of transfection, the cells were treated with CDCA diluteded from 50 μmol/L to 0.05 μmol/L.

and the maximum fold induction of luciferase activity (Fig. 7) by CDCA was higher than 10 at CDCA of 50 μmol/L. The EC_{50} of CDCA was 3.2 μmol/L.

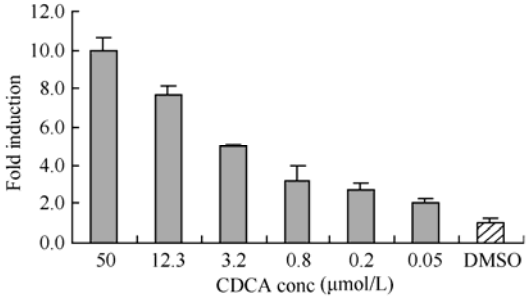

FIG. 7. The effect of CDCA on the fold induction of luciferase activity. After 6 hours of transfection, the cells were treated with CDCA diluted from 50 μmol/L to 0.05 μmol/L.

Using such a transfection condition, about 4000 random samples were screened, and about 0.1% of two samples were found to activate the transcription in a dose-dependent manner (data not shown). The Z′-factor values (Fig. 8) for the screened plates were on average higher than 0.65 and inter-plate correlation variations (CVs) of 96-well plates were less than 6%.

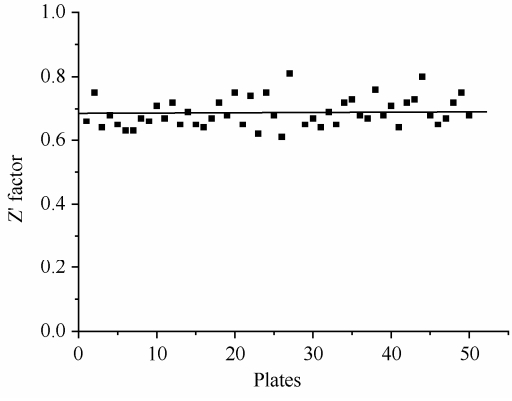

FIG. 8. Reproducible performance—Z′-factor values obtained in each plate by screening 2000 samples for FXR agonist in 96-well plates with positive CDCA (50 μmol/L) and negative DMSO (0.5%).

DISCUSSION

Mammalian one-hybrid system, also known as Chimera receptor gene system, is a functional transactivation cell-based assay system[9-10]. Such an assay system allows the user not only to detect hits, but also to determine the biological function of compounds. Mammalian one-hybrid system has one major advantage that can eliminate interference

factors of mammal cell endogenous nuclear receptors for using the yeast GAL4 response element in mammalian cells, and can lower the noise value of the assay.

FXR has become a valuable target for the identification of novel drugs to treat dyslipidaemia and cholestasis. To obtain new active compounds from the natural or chemical library that can activate the function of FXR, we developed a HTS screening model of FXR agonists based on mammalian one-hybrid system. The sensitivity and stability of a reporter gene assay largely depend on the luciferase signal and S/N ratio. When constructing the reporter vector, we selected a strong promoter in reporter vector by inserting five copies of GAL4 DNA binding site into upstream of the SV40 promoter, which resulted in a high luciferase signal and a high S/N ratio.

Because many factors, such as volume of liposome, amount of DNA, molar ratio of reporter vector to expression vector, would significantly affect the efficiency of cotransfection, and impact the outcome of HTS, the transfection condition must be optimized. After optimization, the S/N ratio of this FXR HTS assay was improved from 5.6 to 11.4.

Z' factor is a statistical function used commonly to judge a high-throughput screening assay robust[11], because the formula for Z' factor, standard deviation (SD), as well as the difference in the means of high and low assay values are taken into account. For cell-based assays, in general, signal/noise ratio should be at least 2, Z'-factor should exceed 0.4, and inter-plate correlation variation of 96-well plates should not exceed 15%. In our screening assay system, CDCA, the natural FXR agonist, could induce expression of the luciferase gene in a dose-dependent manner with the Z' factor value over 0.65, the S/N higher than 10, and the inter-plate correlation variation lower than 6.

In conclusion, the cell-based assay is stable, sensitive and reproducible.

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