Method for Screening Ecdysone-Inducible Stable Cell Lines

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ABSTRACT

Ecdysone-inducible systems are useful tools to study the function of various genes in different types of mammalian cells. However, it is technically difficult to establish stable cell lines. This is partly because the conditional expression system requires the expression of two or more components driven by different kinds of promoters. In this paper, we describe the use of a luciferase reporter gene system for rapid screening of cell lines that express the ecdysone and retinoid X receptors. Using this system, two human stable colon cancer cell lines, SW480/VgRXR and HCT116/VgRXR, were successfully generated. The expression of these receptors remained high after six months of continuous culturing. A tight regulation of gene induction in SW480/VgRXR was observed using 2 μ M Ponasterone A. The gene induction was rapid and persistent. Our results demonstrated the advantage of establishing cell lines that continuously express high levels of ecdysone receptor proteins.

INTRODUCTION

The ecdysone-inducible system is one of several commercially available exogenous gene-inducible systems for mammalian cells (Invitrogen, Carlsbad, CA, USA). In the presence of ecdysone or its analog, heterodimeric retinoid X receptor (RXR) and ecdysone receptor (VgEcR), both encoded in the pVgRXR vector, form a complex and bind to the ecdysone response element in the pIND expression vector. Expression of the target gene is thereby transactivated.

Despite the well-designed mechanism, there remain technical difficulties in the process of establishing stable ecdysone-inducible cell lines such as leakage of target gene expression and/or low or no induction of gene expression. The latter can be due to low expression of either the receptors or the transgene. It is difficult to determine which factor is responsible for low expression if all components are transfected simultaneously. To more efficiently generate cell lines, it is advantageous to first establish a cell line that stably expresses the receptor proteins RXR and VgEcR proteins. However, so far, no rapid, sensitive, and quantitative screening system is available.

To facilitate the identification of cells that functionally express the ecdysone receptor, Invitrogen supplies a lacZ-based reporter system, pIND/lacZ, that exhibits β -galactosidase (β -gal) activity within 2 h of staining. We also performed a quantitative chemiluminescent β -gal assay in which light emission is measured in a luminometer. However, we were unable to identify highly expressing receptor cell lines, probably due to high background from endogenous enzymatic activities pre-

sent in the β -gal system. Therefore, we could not completely exclude leakage and low induction of gene expression. Moreover, the β -gal assay is not a rapid screening system because of its long reaction time of up to 60 min. Thus, we generated a firefly reporter gene assay method to rapidly identify highly expressing ecdysone and retinoid X receptors cell lines. A luciferase reporter system has low background to signal because no endogenous bioluminescence is present. The reaction time was reduced to 15 s. In addition, the reporter performance can be normalized to the transfection efficiency using a control reporter that is not available for chemiluminescent β -gal assay systems. Using a firefly reporter gene assay system, we successfully established two colon cancer cell lines that stably express receptors for more than six months of continuous culture. In this paper, we elucidated their inducibility and other characteristics.

MATERIALS AND METHODS

Plasmid Construction

The pGL2/Enhancer and pGL3/Basic firefly reporter gene plasmids, herpes simplex virus thymidine kinase (TK) promoter-driven *Renilla* luciferase plasmid pRL/TK and CMV promoter-driven GAL4 cDNA coding plasmid pBIND, were purchased from Promega (Madison, WI, USA). The ecdysone receptor coding plasmid pVgRXR and expression vector pIND plasmid were purchased from Invitrogen. To generate the ecdysone-inducible firefly luciferase reporter genes pGL2/EcR and pGL3/EcR, the ecdysone receptor re-

Table 1. Initial Screening of Ecdysone Receptor-expressing cells: pGL2/EcR Reporter Gene Induction

SW480/VgRXR Cells		HCT116/VgRXR Cells	
Cell Line	Fold Induction	Cell Line	Fold Induction
2	6.8	<u>1</u>	<u>22.8</u>
<u>3</u>	<u>69.9</u>	6	12.8
<u>6</u>	<u>81.6</u>	10	15.0
13	16.4	18	11.4
14	3.6	<u>21</u>	<u>54.6</u>
15	3.6	<u>24</u>	<u>21.8</u>
16	10.6	27	10.9
<u>20</u>	<u>88.6</u>	28	10.6
<u>24</u>	<u>85.9</u>	<u>31</u>	<u>25.0</u>
26	3.8	37	12.0
27	3.5	<u>38</u>	<u>27.1</u>
32	3.1	41	6.6
38	3.4		
44	2.2		

Highly expressing lines are underlined.

sponsive element was excised from the pIND plasmid using restriction enzymes *Bgl*II and *Hind*III (New England Biolabs, Beverly, MA, USA). Subsequently, the fragment was subcloned into the *Bgl*II/*Hind*III sites of the pGL2/Enhancer or pGL3/Basic plasmids. For the pIND/Luc plasmid construction, the firefly luciferase cDNA was excised from the pGL3/Basic plasmid using *Bgl*II and *Xba*I and subcloned into the *Bam*HI/*Xba*I sites of the ecdysone expression vector pIND plasmid. EGFP cDNA was excised from the pp53EGFP plasmid (Clontech Laboratories, Palo Alto, CA, USA). Subsequently, pIND/EGFP was generated by subcloning the cDNA fragment into the *Bam*HI/*Xba*I sites of the pIND plasmid.

Establishment of SW480/VgRXR and HCT116/VgRXR Cell Lines

SW480 and HCT116 human colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). SW480 cells were cultured in phenol red-free Leibovitz's L-15 medium supplemented with 10% heat-inactivated FBS (both from Life Technologies, Rockville, MD, USA) in a 0.8% CO₂ incubator at 37°C. HCT116 cells were maintained

in phenol red-free RPMI 1640 medium (Life Technologies) supplemented with 10% FBS in a 5% CO₂ incubator at 37°C. One microgram of pVgRXR (Invitrogen) plasmid encoding the ecdysone receptor was transfected into 1 × 10⁵ SW480 or HCT116 cells, respectively, using FuGENE™ 6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. After 24 h incubation, 1 mg/mL Zeocin (Invitrogen) was added to the medium and cultured for an additional two weeks. Individual colonies were trypsinized, seeded, and expanded. A sample of each clone was transfected with pGL2/EcR and cultured for 48 h in the presence or absence of 10 µM Ponasterone A (Invitrogen). In total, 50 SW480/VgRXR and HCT116/VgRXR clones were picked, and their inducibility was measured using the Dual-Luciferase Reporter Assay System (Promega). The clones demonstrating the highest inducibility were used for further studies.

Inducibility of SW480/VgRXR and HCT116/VgRXR Cells

Cells (2.5 × 10⁴) were seeded into each well of a 24-well plate. After 24 h, cells were co-transfected with 200 ng

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each inducible reporter gene plasmid and 50 ng TK promoter-driven *Renilla* luciferase reference plasmid (pRL/TK) using the TransFast™ lipofection kit (Promega) according to manufacturer's recommended protocol. All transfections were performed in triplicate. Cultured for 24 h in the presence of various concentrations of Ponasterone A or dimethyl sulfoxide (DMSO). Cells were then lysed with 100 μ L lysis buffer (Dual-Luciferase Reporter Assay System), and 30 μ L lysate were assayed for the luciferase activity using Micro Lumat Plus™ LB96V (Wallac/Belthold, Wellesley, MA, USA).

Immunoblotting

Cells (2×10^5) were seeded into the 6-well dishes and cultured for 24 h. Subsequently, cells were transfected with 1 μ g pIND/EGFP and cultured in the presence or absence of Ponasterone A for 24 h. CMV promoter-driven GAL4 cDNA coding plasmid (pBIND) (0.5 μ g) was co-transfected in each sample as an internal control for transfection efficiency. Cells were washed twice with PBS and lysed in 200 μ L SDS-PAGE sample buffer (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysate samples (10 μ L) were separated on a 4%–20% acrylamide linear gradient SDS-PAGE gel (Bio-Rad Laboratories), transferred onto nitrocellulose membrane, and blotted with an anti-GFP and anti-GAL4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signals were visualized using the ECL® kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

RESULTS

After selection with Zeocin for two weeks, 50 independent colonies from heterodimeric ecdysone receptor-transfected SW480 and HCT116 cells were screened with our pGL2/EcR reporter plasmid and the Dual-Luciferase Reporter Gene Assay (Table 1). Four and five clones highly expressing the ecdysone receptors were obtained from SW480 and HCT116, respectively (SW480/VgRXR and HCT116/VgRXR). The highest inducible clones from SW480/VgRXR and HCT116/

VgRXR showed about 90- and 55-fold induction, respectively, after 24 h treatment with 10 μ M Ponasterone A. We continuously cultured the cells (SW480/VgRXR no. 20 and HCT116/VgRXR no. 21) to ascertain stable expression.

After six months, these cells maintained high inducibility (Figure 1, A, B, C, and D). However, the magnitude of the inducibility was reduced. SW480/VgRXR and HCT116/VgRXR cells showed about 20- and 10-fold induction, respec-

Figure 1. Reporter gene induction in ecdysone receptor-expressing SW480/VgRXR cells and HCT116/VgRXR cells. Cells were transfected with pGL2/EcR, pGL3/EcR, or pIND/Luc and cultured for 24 h in the presence or absence of Ponasterone A. TK promoter-driven *Renilla* luciferase plasmid (pRL/TK) was co-transfected in each sample as an internal control for transfection efficiency. The ecdysone receptor responsive element from the pIND ecdysone expression vector was subcloned into either pGL2/Enhancer or pGL3/Basic firefly luciferase reporter plasmid (pGL2/EcR or pGL3/EcR). Luciferase cDNA was subcloned into the pIND expression vector (pIND/Luc). (A and B) SW480/VgRXR cells. (C and D) HCT116/VgRXR cells. (A and C) Comparison of the inducibility. Cells were transfected with pGL2/EcR, pGL3/EcR, or pIND/Luc, respectively. Firefly luciferase activity was normalized against *Renilla* luciferase activity in SW480/VgRXR cells. (B and D) Induction of firefly luciferase activity by pGL2/EcR, pGL3/EcR, or pIND/Luc and reference *Renilla* luciferase activity by pRL/TK. (E) Immunoblot analysis of EGFP induction in SW480 cells. Cells were transfected with 1 μ g pIND/EGFP and cultured in the presence or absence of Ponasterone A for 24 h before immunoblotting. CMV promoter-driven GAL4 cDNA coding plasmid (pBIND) (0.5 μ g) was co-transfected in each sample as an internal control for transfection efficiency.

tively, as measured by the same pGL2/EcR reporter gene after treatment with 10 μ M ecdysone analogue Ponasterone A for 24 h (Figure 1, A and C).

In addition, we tested three different kinds of backbone vectors that drove

the same luciferase gene. SW480/VgRXR transfected with either pGL2/EcR or pIND/Luc reporter plasmids showed a more than 20-fold induction after treatment with 2 μ M Ponasterone A, whereas HCT116/

Figure 2. Prompt and efficient response of SW480/VgRXR cells to Ponasterone A. (A) SW480/VgRXR cells treated with Ponasterone A responded in a dose-dependent manner. Cells were co-transfected with the inducible plasmid and the reference plasmid pRL/TK and cultured for 24 h in the presence or absence of Ponasterone A. Firefly luciferase activity by inducible plasmids was normalized against *Renilla* luciferase activity in SW480/VgRXR cells. (B, C, and D) Time course of induction. Cells were co-transfected with the pGL3/EcR and pRL/TK plasmids in the presence of 2 μ M Ponasterone A and collected at the indicated time points up to 24 h. (B) pGL3/EcR plasmid-driven firefly luciferase activity normalized against pRL/TK-driven *Renilla* luciferase activity. (C) Comparison of inducible firefly luciferase activity and reference *Renilla* luciferase activity. (D) Immunoblot analysis of EGFP induction in SW480 cells. Cells were transfected with 1 μ g pIND/EGFP cultured in the presence or absence of Ponasterone A for 24 h before immunoblotting. CMV promoter-driven GAL4 cDNA coding plasmid (pBIND) (0.5 μ g) was co-transfected in each sample as an internal control for transfection efficiency.

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VgRXR cells only showed 2–2.5-fold induction. According to these results, SW480/VgRXR cells seemed to have a 10 times better response to the same amount of Ponasterone A. However, the third reporter plasmid used in this assay, pGL3/EcR, demonstrated higher inducibility (Figure 1, A and C, and Figure 2A). Especially in SW480/VgRXR cells, the better inducibility by pGL3/EcR appeared even at lower concentrations than 2 μ M Ponasterone A. The induction level of SW480/VgRXR by pGL3/EcR reporter was about 100 times higher than that of HCT116/VgRXR cells. There was a significant difference in the inducibility of pGL3/EcR compared to the other reporter constructs in SW480/VgRXR cells (Figure 1, A and B). Actual firefly luciferase activity measurements indicated that the difference came from the low background of pGL3/EcR in SW480/VgRXR cells (Figure 1, B and D). The level of inducibility reached a plateau at 2 μ M Ponasterone A for all three vectors. A similar result was observed when the pIND/EGFP plasmid was used on SW480/VgRXR, and EGFP expression was measured by immunoblot (Figure 1E). EGFP was induced by the addition of 2 μ M Ponasterone A but did not show a further significant increase at 10 μ M. Reference *Renilla* luciferase gene expression from pRL/TK was not affected by the Ponasterone A treatment up to 10 μ M (Figure 1, B and D).

We tested the response of SW480/VgRXR cells to lower concentrations of Ponasterone A than 2 μ M to find optimum conditions (Figure 2A). Within the range between 0.5 and 2 μ M Ponasterone A treatment, luciferase activity was increased in a dose-dependent manner. This suggests that the tight regulation of the transgene in SW480/VgRXR cells by Ponasterone A is in the range of 1–2 μ M. Next, a time course study was performed using 2 μ M Ponasterone A (Figure 2B). Gene induction was already observed after 2 h and reached 200-fold induction after 8 h (Figure 2, B and C). The induced expression of firefly luciferase continued up to 24 h. On the other hand, the *Renilla* luciferase activity driven by TK promoter indicated that the transfection efficiency was equal. This observation

was confirmed with protein blot using the pIND/EGFP plasmid onto SW480/VgRXR cells (Figure 2D). The target gene expression was indeed induced promptly and efficiently.

DISCUSSION

Conditional expression systems for proteins in mammalian cells are potent tools for studying the function of a gene and its corresponding protein. Those systems do not require physiological stimulation to activate the target gene. Therefore, the function of the protein can be analyzed without influence of upstream signaling pathways. Establishment of a cell line that allows induction of the target gene expression using these systems makes it possible to study the target gene functions under highly reproducible conditions. Tight regulation of gene expression system makes it possible to induce high levels of the cell death proteins (1) or cell growth-inhibitory molecules (6).

There are several widely used conditional expression systems for mammalian cell lines, in particular the tetracycline-inducible promoter (2), the metallothioneine promoter (5), the Lac repressor system (3), and the ecdysone-inducible system (4). The choice of the system is determined by the sensitivity of the cell lines towards the inducers used. For this reason, the ecdysone-inducible system may not be optimal for hormone-sensitive cell types, such as breast cancer cell lines. This system shows high sensitivity towards the addition of steroid hormones. Except for the metallothioneine promoter system, all the systems consist of two or more components supplied by different plasmid vectors to allow the expression of the target gene by either a repression- or an activation-based method.

From our study, we conclude that it is advantageous to first generate a stable cell line expressing high levels of functional modulator protein. Our new technique based on a widely used luciferase reporter gene assay allows the rapid identification of highly inducible receptor-expressing cells. Furthermore, this method is useful to determine the dynamic range of induction using various concentrations of inducers.

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