GENERATION STABLE CELL LINE USED FOR PROTEIN EXPRESSION SYSTEM

Nguyen Thi Thu Huong

Received: 25 July 2017 / Accepted: 10 October 2017 / Published: November 2017

Abstract: *The ecdysone inducible system was generated for checking the the toxicity of the target gene which was transfected into the cells. The retinoid X receptor (RXR) was transfected into HEK293FT to generate an inducible stable cell line pVgRXR-HEK293FT. For checking the function, the cells transfected with pIND-GFP and induced by 1μM ponasteron A for GFP expression. The fluorescence signals were detected in the transfected and induced cells informed the success of experiment.*

Keywords: *Ecdysone inducible system, ecdysone receptor, pVG-RXR, HEK293FT.*

1. Introduction

In order to generate the target proteins for scientific work, cellular protein expression systems are widely utilized. However, besides other influences, the success of protein expression experiments can be limited due to a toxic side effect of overexpression of target protein. In order to overcome this limitation, inducible expression systems like the glucocorticoid inducible mouse mammary tumor virus (MMTV) system, ecdysone-inducible *Drosophila* analog promoter/receptor (EcP) system and the tetracycline-dependent system (Tet) are commonly used (Meyer-Ficca et al, 2004).

The ecdysone inducible system displays a low basal activity, high inductivity and fast response. And the most important feature is its switch on or off ability depending on certain requirements (Padidam et al, 2003; Meyer-Ficca et al, 2004). In addition, due to the natural lipophilic form, ecdysones are able to penetrate into all tissues and have a short half-live which allow precise and potent inductions (No et al, 1996; Oehme et al, 2005). Moreover, ecdysteroids are not toxic and are not known to affect mammalian physiology (No et al, 1996). Based on these perspectives, the ecdysone inducible promoter system was utilized to induce the expression of different subunits of AChR in this study.

Belonging to ecdysteroid family, ecdysone is the insect molting steroid hormone triggering metamorphosis in insects, for example *Drosophila melanogaster*. An increase of ecdysone concentration in *Drosophila* leads to the expression of genes coding for proteins that are necessary for larva development. The synthesized ecdysone inducible system bases on two

Nguyen Thi Thu Huong
Faculty of Agriculture, Forestry and Fishery, Hong Duc University
Email: Nguyenthithuhuong@hdu.edu.vn (✉)

plasmids. One is a receptor expression plasmid, pVg-RXR containing the modified ecdysone receptor (VgEcR) with the ecdysone binding domain and the retinoid X receptor (RXR). Another is a plasmid containing the ecdysone- responsive IND promoter which is a fusion of the ecdysone responsive elements and minimal heat shock promoter. In general, the binding of ecdysone or its synthesis analog (ponasteron A, munisterone A) to ecdysone binding domain of VgEcR promotes the dimerization of modified ecdyson receptor (VgEcR) and the retinoid X receptor (RXR). The heterodimer afterwards binds to the ecdysone responsible elements in the synthesis ecdysone responsive IND promoter. (Lueers et al, 2000). The interaction results in the activation transcription of target gene fused downstream to minimal heat shock promoter. The principle is shown in Figure 1.

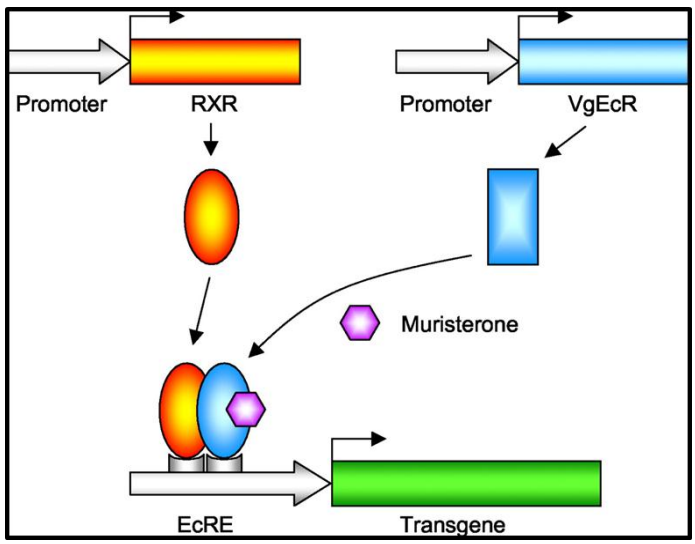

Figure 1. *Schematic diagram of ecdysone-inducible gene expression system*

The inducer (Munisterone) binds to the ecdysone binding domain on the modified ecdysone receptor (VgEcR). This interaction will lead to the hetero-dimerization with retinoid X receptor (RXR). The receptor complex then interacts with the ecdysone responsible elements (EcRE) and acts as the transcription factor and consequently resulting in the activation transcription gene of interest (Figure taken from Luisa Barzon et al, 2004).

However, the requirement to introduce two vectors into the experimental cell system could be the disadvantage of this system. After integration into the genome, transcriptional activity of both constructs could be affected from the flanking genomic sequence resulting in system non- function (Lueers et al, 2000).

2. Materials and Methods

2.1. Materials

HEK293FT cells (Life Technology), pVG- RXR ((Invitrogen), pIND- GFP (Addgene).

The chemicals used in this research were of high quality of Merck (Germany), Sigma - Aldrich (Germany), Invitrogen (USA), Carl Roth (Germany), Fermentas (Germany), VWR (Germany).

2.2. Methods

2.2.1. Cell culture

HEK293FT cells were cultivated in Dulbecco's Modified Eagle Medium (D- MEM) supplemented with 10% fetal bovine serum (FBS), 2mM L- Alanyl - L- Glutamine, 1% non-essential amino acid, 1mM sodium pyruvate and antibiotic G418 (50µg/ ml). The experiments were performed according to Lab manual and SOPs Molecular Cell biology (Kuepper, 2009).

2.2.2. Lipofectamin™ 2000 Transfection

Lipofectamin™ 2000 reagent is a cationic liposome formulation complex with nucleic acid molecule via the ionic interaction. The DNA-Lipofectamin complex overcomes the electrostatic repulsion of the cell membrane and transports into nucleus of the cells (Dalby et al 2004). The procedures were performed according to manual of Lipofectamin™ 2000 reagent (Invitrogen).

Because the HEK293FT cells are not tightly attached to the conventional surface of the cell culture flasks as well as the well plates, the cells were transfected as suspension. The cell seeding number depends on the used plate format. Lipofectamin™ 2000 and DNA were diluted in a serum free medium (table 1). The diluted Lipofectamin was incubated for 5 mins at room temperature (RT) before mixing with the diluted plasmid DNA. The mixture was incubated for 25 mins at RT. The DNA-Lipofectamin complex was subsequently added dropwise in the cells.

Table 1. *Experimental set up for the transfection with Lipofectamin™ 2000*

Plate format	Cell number	Plasmid DNA (µg)	Volume of Lipofectamin (µl)	Volume of serum free medium (µl)
96 well plates	$2x10^4$	0.2	0.5	2x25
24 well plates	$2x10^5$	0.8	2.0	2x50

2.2.3. Generation stable cell line pVgRXR HEK293FT

The receptor expression plasmid pVgRXR was transfected into HEK293FT cells by Lipofectamin™ 2000 reagent in 24 well plates. One day after transfection, the cells were harvested by trypsinizion. Thereafter, the cells were transferred into the 10cms cell culture dishes at three different cell number (10^4, $5x10^4$, 10^5 cells/dish). The cells were then supplemented with 200µg/ml Zeocin for selection. Transfected cells were cultivated

for the clone formation for three weeks. HEK293FT cell culture medium containing Zeocin (200 µg/ml) was changed two times per week. The dishes containing more than 50 colonies were discarded.

2.2.4. Isolation pVgRXR- HEK293FT colonies

The plates containing below 50 colonies were washed one time by 1X PBS. The cloning discs were first incubated on 1X Trypsin/ EDTA for 5 mins at RT and then placed on each clone. The incubation was performed at 37^0C for 10 mins. After that, cloning dishes were transferred to 24 well plates containing HEK293FT cell culture medium supplemented with 200µg/ml Zeocin. Cells were cultured until reaching 80-90% confluence and then harvested by trypsinizion. The cells were seeded into 96 well plates to test their function. The rest of cells were transferred into 25cms T flasks for continuing cultivation.

3. Results

HEK293FT cells were transfected with pVgRXR plasmid in 24 well plates. 24 hours following incubation, the cells were trypsinized and transferred to the 10 cm cell culture dishes at three cell densities including 10^4, $5x10^4$, 10^5 cells/dish, three-time replications. The cells were selected by 200µg/ml Zeocin for three weeks. The dishes containing more than 50 clones were discarded.

Finally, 21 clones were picked (data not shown). In order to check the function, the cells were transfected with pIND-GFP in 96 well plates by using Lipofectamin™ 2000. 24 hours after incubation, the target gene expression was induced by 1µM ponasteron A. The induction was performed during five days. In addition, HEK293FT cells served as the control. The pVgRXR-HEK293FT cells transfected with pIND-GFP without ponasteron A induction and non-transfected pVGRXRHEK293FT cells with ponasteron A treatment, were utilized as the additional controls. Six out of 21 clones showed fluorescence signal after transfection with pIND-GFP and induction with 1µM ponasteron A. Representing for all positive clones, the results for clone 11 and 16 are shown in figure 2.

Moreover, HEK293FT cells transfected with pIND-GFP either with and without ponasteron A induction showed very weak fluorescence signal (IA, IB). As expected, no signal was seen in non-transfected HEK293FT cells with ponasteron A induction (IC). In the case of pVgRXR-HEK293FT cells, a strong green fluorescence signal was seen in the cells transfected with pIND-GFP and induced by 1µM ponasteron A (IIA, IIIA). The cells transfected with pIND- GFP and without ponasteron A induction, no signal was detectable in two out of six clones (clone 11-II and clone 17- data not shown) (IIB). In contrast, a few signals were detectable in other four clones (clone 16 -IIIB). Surprisingly, a few signals were also seen in several non-transfected pVgRXR-HEK293FT cells with 1µM ponasteron A induction (IIC, IIIC).

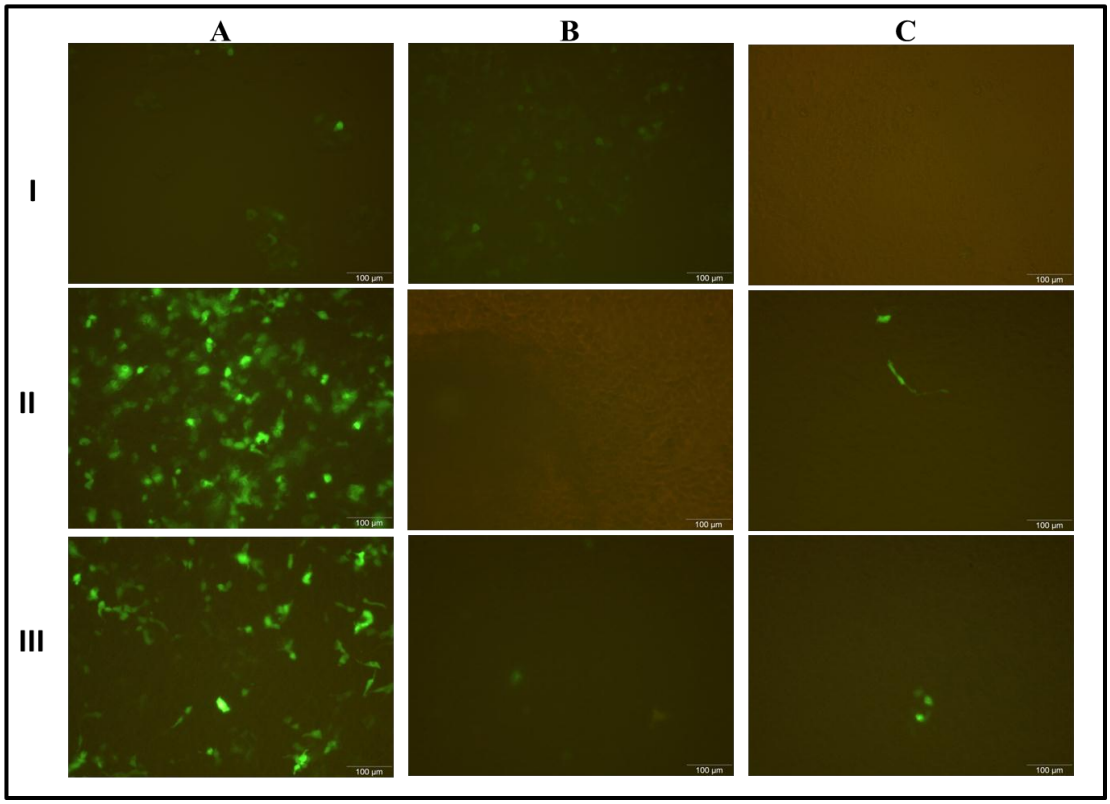

Figure 2. *Characterization the function of stable expressing receptor cell line, pVGRXR HEK293FT*

The HEK293FT cells were suspension transfected with pVgRXR in 24 well plates using Lipofectamin™ 2000. Following 24 hours after transfection, the cells were trypsinzed and transferred into the 10 cms cell culture dishes at three cell concentrations including 10^4, $5x10^4$, 10^5 cells/ dish. The cells were selected by 200µg/ml zeocin. Cells were incubated for clone formation for three weeks. 21 pVGRXR-HEK293FT clones were selected (data not shown). Their function was analyzed by transfecting with pIND-GFP in 96 well plates by Nanofectin (chapter 4.6.2). The cells were incubated for 24 hours and then induced with 1µM ponasteron A. The GFP expression was analyzed 24 hours after post induction.

Lane I: HEK 293FT cell, Lane II: pVgRXR HEK293 FT clone 11, Lane III: pVgRXR HEK293FT clone 16. Lane A: the cells transfected with pIND-GFP with ponasteron A induction, Lane B: the cells transfected with pIND-GFP without 1µM ponasteron A induction. Lane C: the non- transfected with 1µM ponasteron A induction.

The GFP expression was weak in the transfected HEK293FT cells with (IA) and without (IB) ponasteron induction. No signal was detected in non- transfected HEK293FT cell with ponasteron A induction (IC). pVGRXR HEK293FT clone 11 (IIA) and clone 16 (IIIA) were strong in GFP expression. Transfected cells without ponasteron induction showed no GFP expression in case of clone 11 (IIB) or very weak GFP expression in case of clone 16

(IIIB). pVgRXR HEK293FT cells without pIND-GFP but with ponasteron A showed surprisingly some GFP positive cells (IIC, IIIC). Pictures were taken at fluorescence microscope CKX41; Scale bar: 100µm; Expose time: 2.0s. fluorescence microscope CKX41 48hours after induction. Expose time: 2.0s. Scale bar: 100µm.

4. Discussion

HEK293FT cell line (human embryo kidney) is a fast-growing, highly transfected clonal isolate derived from human embryo kidney cells and transformed with the SV40 large T antigen. Expression of the SV40 large T antigen is controlled by the human *cytomegalo virus* (CMV) promoter (Thomas et al, 2004). Special characteristic of HEK293FT cells is not tightly attached to the conventional surface of the cell culture flasks as well as the well plates. At 90% cell confluence, the cells are detached and floated in the cell culture medium.

In order to analyse the cell transfected efficiency, cells were harvested after 48hrs post-transfection, washed, and then resuspended to PBS containing 0.02% EGTA and 1 µg/ml propidium iodide to identify the nonviable cells through propidium iodide fluorescence. Afterwards, the cells were sorted by flow cytometry, evaluated with the Cellquest software to determine the proportion of fluorescent cells. However, the main purpose of project was only generalize inducible cell line to over come the limitation of target protein's overexpression, this step was skipped.

To select a stable cell line pVgRXR-HEK293FT, the cells were treated with 200µg/ml Zeocin for selection. After three weeks cultivation, 21 clones (named 1-21) were isolated in which six clones (2, 11, 14, 16, 17, 21) showed fluorescence signal after transfection with pIND-GFP and induction with 1µM ponasteron A. This matched with the finding of Lueers et al (2000). They transfected pVG-RXR and pER-EGFP into CHO (Chinese hamster ovary) cells and incubated for clone formation and selected by 100µg/ml Zeocin. The isolated clone showed fluorescence signal of GFP after induction with 1µM ponasteron A.

At the begining, the cells were cultivated at three cell densities including 10^4, $5x10^4$, 10^5 cells/dish, repeated three times and after three week cultivation, six functional clones were isolated. Assuming that at the ideal model, each clone was formed from one transfected cell, cloning efficiency was 0.00125%. However, due to the special characteristic of HEK cells, these clones could be also formed by the gathering of floating cells. In this case, cloning efficiency could not be calculated.

The protein expression level was estimated through the GFP signal at the treatment of transfection with pIND-GFP and induction with 1µM ponasteron A. Fluorescence signal indicated that protein expression of clone 11 and 17 was about 85%, clone 14 and 16 was about 50% and clone 2 and 21 was about 30%.

A few cells with weak fluorescence signal were seen in the HEK293FT cells transfected with pIND-GFP either with or without ponasteron A induction. This result demonstrated the GFP expression under normal condition. In addition, four out of six clones showed a very

weak fluorescence signal after transfection with pIND-GFP but without ponasteron A induction (see figure 2). The result indicated the leakiness of this inducible system. Even very small amounts of target gene is expressed could result in exponential increase after certain replication cycles. Due to this fact, these clones were eliminated. Other two clones which did not show the signal after transfection with pIND-GFP without 1µM ponasteron A induction were selected for further investigation. The result suggested that the ecdysone inducible system was the useful tool for controlling the target gene expression.

5. Conclusion

An inducible stable cell line was successful generated. For further research, two clones were selected to re-check the functions and use for gene of interest expression.

References

[1] David No, Tso - Pang Yao, and Ronald M. Evan (1995*), Ecdysone - inducible gene expression in mammalian cells and transgenic mice*, Proc. Natl. Acad. Sci. USA, vol.93, pp.3346-3351.

[2] Georg Hermann Lueers, Nicole Jess, Thomas Franz (2000), *Reporter - linked monitoring of transgene expression in living cells using the ecdysone - inducible promoter system*, European Journal of Cell Biology, vol.79, pp.653-657.

[3] I Oehme, S Bo¨sser, and M Zo¨rnig (2006), *Agonists of an ecdysone-inducible mammalian expression system inhibit Fas Ligand- and TRAIL-induced apoptosis in the human colon carcinoma cell line RKO*, Cell Death and Differentiation, vol.13, pp.189-201

[4] Invitrogen (2006), *Lipofectamin™ 2000 user guider*

[5] Küpper JH (2009): *Lab Manual and SOPs Molecular Cell Biology*. Senftenberg

[6] Mirella L. Meyer- Ficca, Ralph G. Meyer. Heike Kaiser, Alexandra R. Brack, Reinhard Kandolf, Jan-Hainer Kuepper (2004), *Comparative analysis of inducible expression systems in transient transfection studies*, Analytical Biochemistry, vol.334, pp.9-19.

[7] Malla Padidam, Michael Gore, D. Lily Lu and Olga Smirnova (2003), *Chemical inducible, ecdysone receptor based gene expression system for plant*, Transgenic Research, vol.12, pp.101-109.

[8] Invitrogen™ life technology (2002), *pcDNA-DEST40 Gateway™ Vector*, available online.

[9] Philip Thomas, Trevor G. Smart (2004), HEK293 cell line: *A vehicle for the expression of recombinant proteins*, Journal of Pharmacological and Toxicological Methods, vol.51, pp.187-200.