

Optimization of Cationic Lipid Mediated Transfection of pEGFP-c1 and pJ-EPO Plasmids in Chinese Hamster Ovary (CHO) Cells Attached Culture for Transient and Stable Recombinant Human Erythropoietin (rhEPO) Expression

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Abstract

Cationic lipid is one of transfection agents which show high efficiency and low cytotoxicity. The transfection efficiencies can be varied depending upon the type or amount of cationic lipids, the cell line or DNA plasmid being used for transfection. The purpose of this study was to find optimal condition for transfection of CHO-K1 and CHO-S cells with pJ-EPO plasmid (containing *human erythropoietin/hEPO* gene) compared with pEGFP-c1 plasmid (containing *green fluorescence protein/gfp* gene) by cationic lipid Lipofectamin 2000TM (lipofectamin) to generate stable transfectant expressing recombinant human erythropoietin (rhEPO). Optimization was carried out regarding the amount of lipofectamin, DNA concentration, and concentration of antibiotic Geneticin (G418) for selection of stable transfectants. By using standard amount of lipofectamin (10 µl/well) in 6-well plate, highest expression level of green fluorescent protein (GFP) was shown after transfection of CHO-K1 cells with 3 µg/well pEGFP-c1 while highest expression level of rhEPO was observed after transfection of CHO-K1 cells with 6, 8, or 10 µg/well pJ-EPO plasmid. The data also indicated that optimal transfection conditions of CHO-K1 and CHO-S cells with pJ-EPO were shown with the use of 4 µg/well DNA in combination with 15 µl lipofectamin. Concentration of G418 used during cells selection also affected the expression where strongest rhEPO expression was shown at 750 ng/µl G418 concentration. Similar to GFP expression profile, rhEPO signal was detected very low during selection process based on Western blot data at day 9. Stronger rhEPO signal was observed after day 20 when the stable transfectants have been obtained.

Key words: cationic lipid, CHO cells, erythropoietin, green fluorescent protein, transfection

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Introduction

DNA recombinant technology is an important tool in biotechnology (Leader *et al.*, 2008). Engineering of eukaryotic mammalian cells by introduction of foreign DNA has been widely used for study of gene expression, production of functional proteins, study of signal transduction and gene therapy (Backliwal *et al.*, 2008; Loignon *et al.*, 2008). For these purposes, both viral or non viral systems have been developed to enhance the efficiency of transfection. As an alternative to viral vector, plasmid vector has been commonly used to clone and introduce the functional genes into the cells by transfection using chemicals, electroporation, or transduction (Kim & Eberwine, 2010) for

transient or stable expression of gene of interests (Bollin *et al.*, 2011; Ho *et al.*, 2010).

There are several transfection techniques that can be performed by using chemical methods. Calcium phosphate is the cheapest and simplest even though in some cases it shows low transfection efficiency (Girard *et al.*, 2001; Jordan *et al.*, 1996). Other transfection methods such as cationic lipid, DEAE dextran, PEI, activated dendrimer and magnetic nanoparticles mediated transfections (Hunt *et al.*, 2010) show better performance and lower cytotoxicity compared with calcium phosphate (Jones *et al.*, 2010). Among transfection methods, cationic lipid-mediated transfection has been widely used because of its high efficiency instead of its low

cytotoxicity (Maurisse *et al.*, 2010; Kim & Eberwine, 2010).

Based on structures, cationic lipid consists of synthetic and non synthetic lipids. Lipofectamin 2000TM is one of non synthetic and commercially available cationic lipid. It has been used for wide range of cell lines and effectively transfects DNA or RNA. For production of recombinant protein, many efforts have been carried out to obtain high copy number of inserted gene as well as to obtain highly expressing cells. Optimization of transfection conditions begin from optimization of the amount of transfection reagent, DNA concentration, and cell number to increase transfection efficiency (Kim & Eberwine, 2010; Kaiser & Toborek, 2001). With higher efficiency, the chance to generate stable cell transformants expressing recombinant protein of interest will be greater.

The purpose of this study was to investigate relationship between Lipofectamin 2000TM as a cationic lipid based transfection agent, DNA concentration and transfection efficiency to obtain high expression of recombinant human erythropoietin (rhEPO) compared with green fluorescent protein (GFP) both in transient and stable transfected CHO cells. For stable transfection, CHO cells were incubated in a complete culture medium containing genetycin (G418) and observation was done until day 20 after transfection.

Materials and Methods

Cell culture and reagents. CHO-K1 and CHO-S cells were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. CHO-K1 cells were grown in F-12 medium (Sigma) and CHO-S were grown in DMEM medium (Sigma), both media were supplemented with 10 % of fetal bovine serum (Sigma) and 100 U/ml of penicillin-100 µg/ml of streptomycin (Invitrogen) in CO₂ incubator with 5 % CO₂ at 37 °C. Plasmid pEGFP-c1 (Clontech) was obtained from Prof. Kawaichi and pJ-EPO was obtained from DNA 2.0. Cationic lipid Lipofectamin 2000TM was purchased from Invitrogen and antibiotic genetycin (G418) was from Sigma.

Preparation of plasmids. Both pEGFP-c1 and pJ-EPO plasmids were transformed into *Escherichia coli* DH-5α. Transformed bacterial cells were grown in 200 ml LB medium and the plasmids were isolated by using Qiagen maxi prep according to the manufacturer's protocol. Isolated plasmids were analyzed by agarose DNA electrophoresis and spectrophotometry for relative quantification of DNA.

Transfection of CHO cells. CHO cells were grown in 25 cm² flask or 10 cm dish in standard culture condition. After at least two passaging times, the cells were harvested by trypsinization and 200,000 cells/ml were seeded into 6-well plate. The next day, transfections were done as follows: for pEGFP-c1 plasmid, one 6-well plate of CHO-K1 cells were transfected with 1, 2, 3, and 4 µg DNA/well with addition of 10 µl Lipofectamin 2000TM/well diluted in F12 basal medium. For pJ-EPO plasmid, two 6 well plate of CHO-K1 cells were transfected with 0, 3, 4, 6, 8 and 10 µg DNA/well with addition of 10 µl Lipofectamin 2000TM/well and 4 µg DNA/well with addition of 0, 5, 10, 15, and 20 µl Lipofectamin 2000TM diluted in F12 basal medium. For CHO-S cells, one plate of cells were transfected with 0, 2, 3, 4, and 5 µg DNA/well with addition of 15 µl Lipofectamin 2000TM in serum free optemem medium. Transfections were done for 6 hours in medium supplemented with FBS, without addition of antibiotics. At the end of transfections, cells were washed with 500 µl basal medium twice and supplemented with fresh medium. GFP was observed 48 h after transfection by using fluorescent microscope. For pJ-EPO transient transfection, cells were incubated for 48 hours and culture medium was collected for further analysis of rhEPO by Western blotting. For stable transfection, cells were passed with passage ratio 1:10 in 6 well plate (about 50,000 cells/well) after overnight incubation, and G418 was added in the medium. Cells were passed every time they reached confluency. G418 was added at final concentration 250, 500, 750, 1,000 and 1,250 ng/µl respectively.

Purification and detection of rhEPO. Culture medium was collected and centrifugated 3000 rpm for 5 minutes. As much as 2 ml of

medium was used for purification. One hundred microlitre sample buffer (500 mM imidazole) and 20 μ l Ni-NTA agarose beads (Qiagen) were added to the medium and then it was incubated in a rotator for about 2-4 hours at 4 °C. At the end of incubation, the suspension was spun at 2,000 rpm and the pellet was washed twice with 1 ml washing buffer. The 6 his-tagged-rhEPO then was eluted with 40 μ l elution buffer.

Western blot. Each of 15 μ l eluates were added with 5 \times SDS loading buffer and subjected into an SDS-polyacrylamide gel. After electrophoresis, the proteins then were wet-transferred into a nitro-cellulose membrane (GE Healthcare) then followed by membrane blocking in 5 % of skim milk in tris buffer saline (TBS). After washing with TBS containing 0.1 % tween-20 (TBS-T), the membrane was probed with rabbit polyclonal anti-human EPO antibody (1:1000; Calbiochem). After the next washing step, the membrane was probed with alkaline

phosphatase conjugated anti-rabbit antibody (2:10,000; Promega). Nitro blue tetrazolium chloride-5-bromo-4-chloro-1H-indol-3-yl dihydrogen phosphate (NBT-BCIP; Promega) substrate was used to visualize the EPO signal. All Western blot images were semi-quantitatively analyzed by using UN-SCAN-IT and presented as percentage of total pixel.

Results

We examined transfection efficiency of CHO-K1 cells with pEGFP-c1 plasmid by using Lipofectamin reagent. The transfection agent was used according to manufacturer's protocol or 10 μ l/well in each well of 6-well plate while DNA concentration used varied from 1 to 4 μ g/well. Expression of GFP after incubation for 48 hours indicated that DNA concentration affected transfection efficiency (Figure 1). During this experiment, transfection by using Lipofectamin 2000TM showed no observable cytotoxicity.

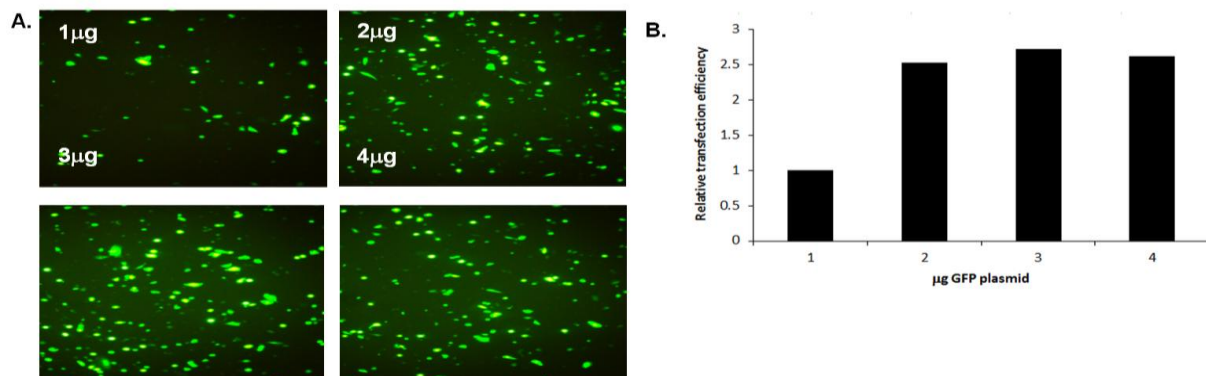


Figure 1. GFP expression in CHO-K1 cells. Observation of GFP expression 48 hours after transfection with 1, 2, 3 and 4 μ g pEGFP-c1 plasmid as indicated (A) and semi-quantitative analysis of transfection efficiency by using ImageJ software (B).

In experiment with pJ-EPO plasmid, expression of recombinant erythropoietin (rhEPO) was observed after 48 hours of incubation following transfection. The result showed that rhEPO expression was detected at DNA concentration of 6, 8, and 10 μ g/well with no significant difference (Figure 2). In further experiment, the optimal amount of lipofectamin for the transfection was examined using DNA concentration of 4 μ g/well and addition of 0, 5, 10, 15 and 20 μ l/well lipofectamin, respectively. Transfection with 10 μ l lipofectamin and DNA concentration of

6 μ g/well was used for comparison. The result indicated that transfection condition for pJ-EPO plasmid by using 4 μ g DNA/well and 15 μ l lipofectamin was better than using 6 μ g/well DNA and 10 μ l lipofectamin (Figure 3). Similar experiment was carried out in CHO-S cells using DNA concentration of 0, 2, 3, 4 and 5 μ g/well, respectively, and addition of 15 μ l lipofectamin. The result of Western blot analysis showed that the highest expression of rhEPO was obtained by transfection with DNA concentration of 4 μ g/well (Figure 4).

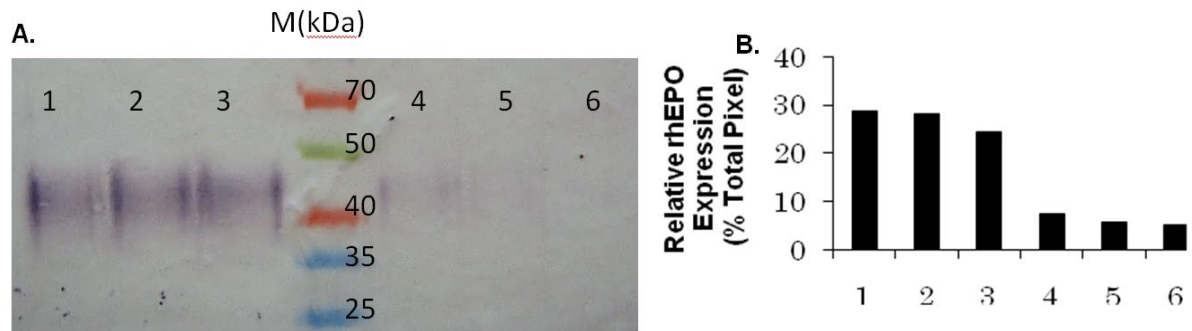


Figure 2. Expression of rhEPO in CHO-K1 after transfection with various concentration of pJ-EPO plasmid. CHO-K1 cells were seeded at density of 4×10^5 cells/6-well plate and transfected with 10, 8, 6, 4, 3, and 0 μg of pJ-EPO plasmid (lanes 1-6). Following transfection, cells were incubated in CO_2 chamber under standard condition for 48 hours. Culture medium was collected, purified by using Ni-NTA agarose beads and detected by Western blot. M: protein marker (A) and semi-quantitative analysis of expression by UN-SCAN-IT software (B).

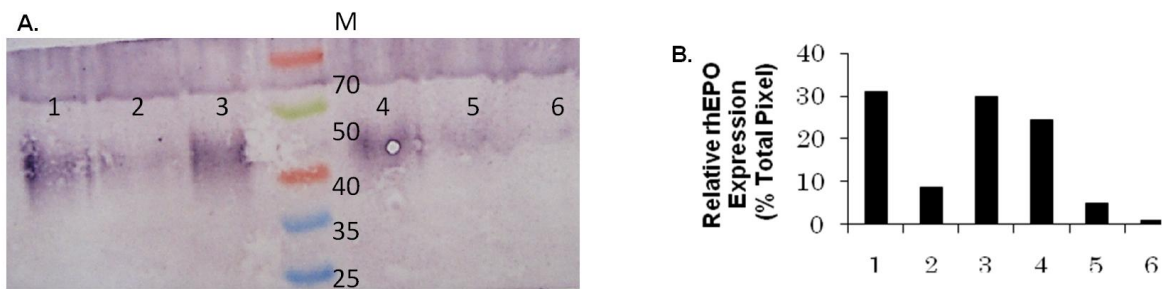


Figure 3. Expression of rhEPO in CHO-K1 after transfection with pJ-EPO plasmid and various amount of Lipofectamin. CHO-K1 cells were seeded at density of 4×10^5 cells/6-well plate and transfected with 6 μg of pJ-EPO plasmid and 10 μl lipofectamin (lane 1), 4 μg of plasmid and 20, 15, 10, 5, 0 μl lipofectamin, respectively (lanes 2-6). M: protein marker (A) and semi-quantitative analysis of expression by UN-SCAN-IT software (B).

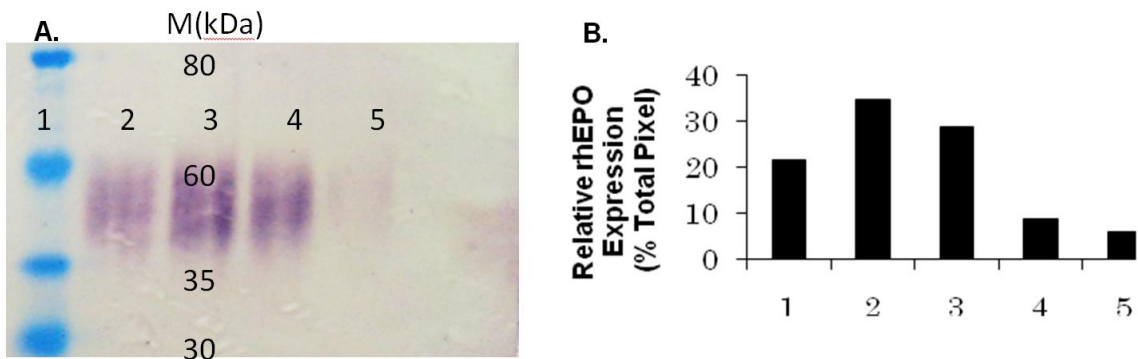


Figure 4. Expression of rhEPO in CHO-S after transfection with various concentration of pJ-EPO plasmid. CHO-S cells were seeded at density of 4×10^5 cells/6-well plate and transfected with 5, 4, 3, 2 and 0 μg DNA/well (lanes 1-5) and 15 μl lipofectamin. M: protein marker (A) and semi-quantitative analysis of expression by UN-SCAN-IT software (B).

Both pEGFP-c1 and pJ-EPO plasmids encode antibiotic resistance gene for neomycin. According to this information, a neomycin analogue, genetycin (G418), was added to culture media for selection of stable transfectant for approximately 20 days. From previous transfection of CHO-K1 cells with 2

μg /well pEGFP-c1, expression level of GFP was decreased after cell passages (Figure 5A). However, after day-20, observable GFP expression was detected (Figure 5B). Similar to GFP expression, rhEPO signal was detected very low by Western blot at day-9, but after 20 days, strongest rhEPO signal was observed

(Figure 5C). Additionally, the concentration of G418 used during selection of stable transfectant also affected rhEPO expression

level. Strongest signal of rhEPO was detected by Western blot after selection of transfectant by using 750 ng/μl G418 (Lane 3, Figure 5C).

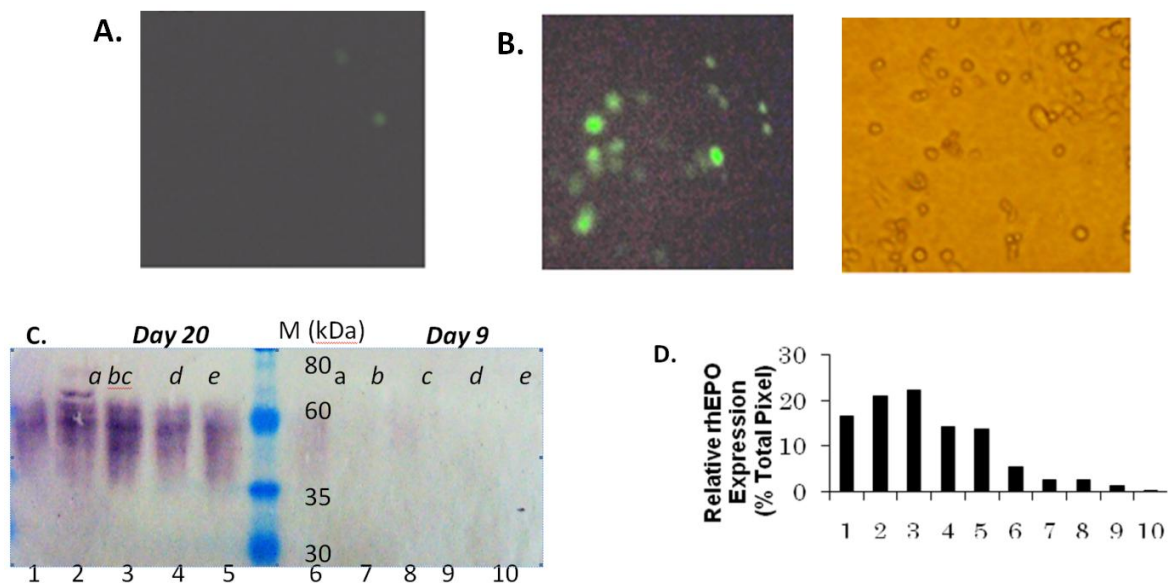


Figure 5. Expression of green fluorescent protein (GFP) and recombinant human erythropoietin (rhEPO) after cell selection by using genetycin (G418). Expression of GFP at 7 days (A) and 20 days (B) after transfection. Cell selection was performed by using 1,000 ng/μl G418. Expression of rhEPO at 9 and 20 days after transfection (C). Cell selection was performed by using 250, 500, 750, 1,000 and 1,250 ng/μl G418, respectively (lanes a-e). M: protein marker. Semi-quantitative analysis of expression by UN-SCAN-IT software (D).

Discussion

The choice of transfection method is an important parameter for transient and stable protein expression. Lipofectamin is categorized as a cationic lipid based transfection reagent. To increase transfection efficiency regarding the use of lipofectamin, amount of lipofectamin, DNA concentration and cells confluency can be optimized dependent upon the cell type (Kaiser & Toborek, 2001). This method works based on rapid interaction between cationic lipid bilayer and anionic polynucleotides such as DNA or RNA to form liposomes (Zasadzinski *et al.*, 2011). The presence of serum interferes with the formation of lipid-DNA complexes because the serum interacts with lipids (Nchinda *et al.*, 2002). The use of appropriate medium to hydrate the cationic lipid will also increase effectiveness of lipid-DNA complex formation (Dalby *et al.*, 2004). Furthermore, enhancement of transfection efficiency can be achieved by controlling the cell cycle phase. Transfection of cells at the G2/M phase or just after mitosis shows better transfection

efficiency than transfection of cells at the G1 phase (Mortimer *et al.*, 1999).

To increase transfection efficiency, cationic lipid should not be used in the presence of serum. It is reported that in the absence of serum, transfection of HeLa and normal human fibroblast cells showed better efficiency (Serikawa *et al.*, 2000). In the case of lipofectamin, it can be used in the presence of serum (Pichet & Ciccarone, 1999). However, synthetic aromatic cationic lipids that have been developed later, show higher transfection efficiency in the presence of serum comparing to lipofectamin (Mochizuki *et al.*, 2012). The advantage of using serum is to minimize cytotoxic effect of transfection on cell growth and viability. In this study, lipofectamin did not show observable cytotoxicity with the use of 10 % serum in medium during transfection.

Lipofectamin reagent can be used either for transient transfection or stable transfection. However, to obtain stable transfection, transfected cells have to be cloned in selection medium (Loignon *et al.*, 2008). The plasmid pEGFP-c1 as well as pJ-EPO has selectable marker for eukaryotic expression system by the

presence of neomycin resistance gene. The gene would express an enzyme which digests neomycin or G418 (Geneticin). Massive cell death was observed in mock control cells in day-20. However, transfected CHO-K1 cells looked healthy, grew well and reached confluency (data not shown). The results indicated the successful transfection of CHO-K1 cells. Without *neomycin resistance* gene, the cells would not survive. Moreover the expression level of GFP was increased in day 20 (Figure 5 B). The supernatant of pJ-EPO transfected cells examined in day 9 showed low level of expression of rhEPO protein and in day 20 showed increase in the expression level due to increased presence of stable transfection cells expressing rhEPO (Figure 5C).

The trend of decrease and increase expression of GFP and EPO protein is a very interesting phenomenon (Figure 5). After a few weeks, the GFP expression was detected again in more cells. The results suggested that successful transfection should be made later after stable transfection has been reached. In stable transfection system, the gene encoding the protein to be integrated into the genome requires selection procedure (Subramanian & Srienc, 1996). During of selection procedure, the genetic materials can be lost by environmental factors and cell division. The choice of transient or stable transfection depends on the purpose of the research. Transiently transfected genes are only expressed for a shorter period and are not integrated into the genome. In contrast to transiently transfected genes, stably transfected gene allows sustained transgene expression (Kim & Eberwine, 2010). Improvement in the monitoring of transfection efficiency and expression level was commonly done by using one plasmid containing *gfp* gene and gene of interest. By this method, GFP expression represents the expression level of our protein of interest. This method also eases the process of isolation of single transfected cell or the separation of successful transformants (Suzuki *et al.*, 2006).

Conclusion

In this study, higher expression levels of GFP and rhEPO were shown after transfection of CHO-K1 cells with 3 $\mu\text{g}/\text{well}$ of pEGFP-c1

or 10 $\mu\text{g}/\text{well}$ pJ-EPO plasmid, respectively by using 10 μl lipofectamin/well of 6-well plate. In addition, optimal transfection conditions for CHO-K1 and CHO-S cells with pJ-EPO plasmid were obtained with the use of 4 $\mu\text{g}/\text{well}$ DNA in combination with 15 μl lipofectamin. The data indicated that the optimal concentration of G418 used for selection of stable transfectant was about 750 $\text{ng}/\mu\text{l}$. Expression levels of GFP and rhEPO were decreased at the beginning of selection or after cell passages at around day-7 to 9, and were increased and observable after day-20.

Acknowledgement

This study was financially supported by the National Innovation Program (SINAs), Ministry of Research and Technology (Menristek) (grant No. RD-096-2013). The authors thank Prof. Masashi Kawaichi for providing CHO-K1 cells, CHO-S cells and pEGFP-c1 plasmids and Ms. Larasati for kind assistance during this study.

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