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Morphological Selection of Parental Chinese Hamster Ovary Cell Clones Exhibiting High-Level Expression of Recombinant Protein

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ABSTRACT

To facilitate the establishment of recombinant Chinese hamster ovary (rCHO) cell lines with dihydrofolate reductase (dhfr)-mediated gene amplification, a primary selection method based on morphology of parental CHO clones has been developed. Morphology of parental clones that were made by transfecting various plasmids encoding thrombopoietin (TPO) and its analogs and humanized antibodies into dhfr-deficient (dhfr⁻) CHO cells was not uniform. Morphology of many parental clones exhibiting high-level expression of the introduced gene was similar to that of nontransfected dhfr⁻ CHO cells. On the other hand, most parental clones with low-level expression experienced noticeable morphological changes such as bipolar fibroblast-like morphology. In case of selection of parental clones with TPO expression level higher than 200 ng/mL, morphological selection improved selection efficiency by 3.5-fold compared with random selection. Furthermore, when subjected to methotrexate for gene amplification, parental clones that were selected based on morphology elevated the expression level as much as those that were selected randomly. Taken together, morphological selection of parental clones can facilitate the establishment of rCHO cell lines expressing recombinant proteins.

INTRODUCTION

For high-level expression of foreign protein, the most popular mammalian expression system in both research and industrial applications uses expression vectors containing the dihydrofolate reductase (dhfr) gene as the selective gene and the dhfr-deficient (dhfr⁻) Chinese hamster ovary (CHO) cells as the host

cell line. For the establishment of CHO cell lines expressing a foreign protein of interest, dhfr⁻ CHO cells are first transfected with expression plasmids including the dhfr and the foreign gene. The widely used nondirected gene transfer method allows expression plasmids to integrate randomly into the genome with different copies and generates a heterogeneous population of cell clones that differ significantly in their expression levels and stability (4,5).

Since the isolation of clones with high-level expression clearly depends on the number of clones that have been screened, time-consuming and tedious screening procedures are required for the isolation of such clones. Thus, there has been an increasing concern for the simplification of screening procedures (3,8,10,11).

In our laboratory, we transfected the thrombopoietin (TPO) expression vector containing the dhfr gene into dhfr⁻ CHO cells for the development of recombinant CHO (rCHO) cells producing a human TPO that is a humoral regulator of megakaryocytopoiesis (1). It was found that the morphology of many parental clones with high-level expression was similar to that of nontransfected dhfr⁻ CHO cells.

In this study, we demonstrate that parental CHO clones with high-level expression of the introduced gene can be screened efficiently based on their morphology, thus facilitating the establishment of rCHO cell lines.

MATERIALS AND METHODS

Vectors

Three different expression vectors were constructed from pSV2-dhfr (37146; ATCC, Rockville, MD, USA) and pCD33r. The pCD33r, which was kindly provided by Daewoong Pharmaceutical (Sungnam, Korea), was built by inserting the cDNA fragment encoding a modified recombinant human TPO into a unique *Kpn*I and *Eco*RI site of pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, USA). For enhancement of gene amplification at low methotrexate (MTX) levels (7), the enhancer element from the simian virus 40 (SV40) early promoter used for the expression of

selectable and amplifiable dhfr in pSV2-dhfr was removed by deleting the 42-bp sequence between the *Sph*I and *Pvu*II sites and joining these sites via a synthetic *Ban*HI linker. The crippled dhfr gene on a 1.6-kb *Ban*HI fragment from pSV2-dhfr was subcloned into the *Bgl*II site of pCD33r. Then, the resulting plasmids, in which orientation of the dhfr expression unit was direct or opposite to that of the cytomegalovirus (CMV) promoter to direct TPO, were selected and named pDr33r-F or pDr33r-R, respectively. The pDr33r vector was identical to pDr33r-F except for further 80-bp deletion in the dhfr promoter region.

Cell Culture, Transfection and Selection

dhfr⁻ CHO cells (CRL-9096; ATCC) were grown and maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine and 10% dialyzed fetal bovine serum (dFBS) (all from Life Technologies, Rockville, MD, USA). Transfection of expression plasmid (2 μ g) into dhfr⁻ CHO cells was performed using LIPOFECTAMINE™ (12 μ g; Life Technologies) according to the manufacturer's protocol. Drug selection was carried out by seeding 1×10^3 cells/well in 96-well tissue culture plates (Nalge Nunc, Roskilde, Denmark) containing IMDM supplemented with 10% dFBS and 500 μ g/mL Zeocin (Invitrogen). Cells were re-fed with fresh medium every 3–4 days for approximately three weeks until the size of the colony reached near confluency. The morphological characteristics of Zeocin-resistant clones isolated on 96-well tissue

culture plates were examined using a phase contrast microscope (Nikon® TMS-F; Nikon, Tokyo, Japan) at a magnification of 100 $\times$. For comparison, non-transfected dhfr⁻ CHO clones were also isolated in 96-well culture plates containing IMDM supplemented with 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine and 10% dFBS, and their morphological characteristics were examined.

The TPO titer in the culture supernatant of Zeocin-resistant clones isolated on 96-well tissue culture plates was determined by an ELISA kit (R&D Systems, Minneapolis, MN, USA). The TPO productivity per cell was determined by dividing the TPO titer in the supernatant by the corresponding OD value ($OD_{570} - OD_{630}$) from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (2).

Gene Amplification of Selected Clones

Based on the TPO titer in the supernatant, Zeocin-resistant clones with high-level expression were selected for gene amplification and transferred to a T-25 cm² flask (Nalge Nunc) containing 5 mL IMDM supplemented with 10% dFBS and 20 nM MTX (Sigma, St. Louis, MO, USA). Cells were re-fed with fresh medium every 3–4 days and were passed upon reaching confluency by trypsinization. After 5–6 passages, cells were seeded at 1×10^5 cells/well in 6-well tissue culture plates (Nalge Nunc). After three days of cultivation, the TPO titer in the culture supernatant of clones subjected to 20 nM MTX was determined by ELISA.

Figure 1. Typical photomicrographs of (A) dhfr⁻ CHO cell clone, (B) characteristic clone and (C) parental clone with distinct morphological change. Photomicrographs were taken at 100 $\times$ magnification.

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Table 1. Statistical Analysis on the Expression Level of Characteristic Clones and Parental Clones

Protein	Plasmid	Average $\pm$ Standard Deviation (ng/mL)				P value ^a		
		Parental Clones	Number of Clones	Characteristic Clones	Number of Clones	Total Clones	Top 20%	Top 10%
TPO33r	pDr33r-F	89.8 $\pm$ 117.3	207	193.5 $\pm$ 159.5	20	0.005	0.004	0.010
TPO33r	pDr33r-R	55.7 $\pm$ 72.7	98	154.9 $\pm$ 99.8	6	0.047	ND	ND
TPO33	pD33r	138.1 $\pm$ 135.6	59	243.9 $\pm$ 174.6	10	0.048	ND	ND
TPO	pDCT	13.1 $\pm$ 24.8	35	50.5 $\pm$ 16.4	4	0.005	ND	ND
Antibody	pKC	142.1 $\pm$ 186.2	40	528.8 $\pm$ 265.9	5	0.013	ND	ND

^aStatistical analysis on parental and characteristic clones with an indicated expression level was carried out using Student's *t*-test with one-tailed test.

ND, not determined (the number of clones is too small to be analyzed statistically).

RESULTS

Morphological Identification of Parental Clones

Parental clones expressing a TPO were isolated on 96-well tissue culture plates after transfecting a TPO expression plasmid (pDr33r-F) into dhfr⁻ CHO. Their morphology on 96-well tissue culture plates was examined by microscopy, and their expression level was determined by measuring the TPO titer in the culture supernatant. Microscopic observation revealed that the morphology of clones was not uniform. As shown in Figure 1, A and B, many clones with high-level expression had morphology similar to that of nontransfected dhfr⁻ CHO cells, though their size was bigger. These parental clones with morphology similar to dhfr⁻ CHO cells were named characteristic clones. On the other hand, most of the clones that experienced distinct morphological changes, such as a bipolar fibroblast-like morphology, displayed low-level expression (Figure 1C), indicating the potential of morphological selection of parental clones with high-level expression.

Efficacy of Morphological Selection of Parental Clones with High-Level Expression

Since not all of the clones with morphological changes displayed low-level expression, the efficacy of morphological selection of clones with high-level

expression was tested. Among a total of 207 parental clones examined morphologically, 20 parental clones were characteristic clones.

Figure 2 shows the expression level of parental clones plotted as a cumulative percentile. Although the expression level of characteristic clones varied significantly, characteristic clones showed relatively high-level expression among 207 clones. Average expression levels of characteristic clones and parental clones were 193.5 and 89.8 ng/mL, re-

spectively (Table 1). Statistical analysis using the Student's *t*-test shows that this difference in the expression level is significant ($P < 0.01$), demonstrating the potential of morphological selection of parental clones with high-level expression. In the case that clones with an expression level higher than 200 ng/mL are selected, selection efficiency in characteristic clones is approximately 35% (7 clones out of 20 clones), while that in a total of 207 clones is less than 10% (20 clones out of 207 clones).

Figure 2. Expression level of parental clones plotted as a cumulative percentile. Closed circle represents a plot for 20 characteristic clones. Open circle represents a plot for 207 parental clones including 20 characteristic clones.

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Accordingly, compared with random selection of clones, preselection based on morphology can improve selection efficiency by 3.5-fold.

When other TPO expression plasmids (pDr33r-R, pD33r or pDCT) and antibody expression plasmid (pKC) were transfected into dhfr⁻ CHO cells, parental clones with high-level expression could also be selected based on morphology. As shown in Table 1, characteristic clones produce a statistically higher level of expression than parental clones regardless of plasmids and proteins. In addition, the percentage of characteristic clones out of parental clones is in the range of 6%–17%, suggesting that this application of morphological selection is not limited to specific expression plasmids.

Since cell growth rate varies among the clones, high-level expression of characteristic clones may be a result of their high cell growth rate. To test this

hypothesis, another set of transfection was performed to estimate the TPO productivity per cell of parental clones. Among the total 36 parental clones examined morphologically, four clones were characteristic clones, and the TPO productivity per cell for them was ranked within the top 8 (data not shown), suggesting that the high-level expression of characteristic clones was not due to high cell growth rate. In fact, the cell growth rate of characteristic clones represented by the MTT value was comparable to that of other parental clones.

Gene Amplification of Parental Clones with Characteristic Morphology

When parental clones with high-level expression are subjected to MTX, they do not always display enhanced expression level. If characteristic clones do not display enhanced expres-

sion level in the presence of MTX, morphological selection is useless for the development of rCHO cell lines exhibiting high-level expression. To verify the efficacy of morphological selection in regard to gene amplification, 12 parental clones, including five characteristic clones, with an expression level higher than 200 ng/mL were subjected to 20 nM MTX. The expression levels of characteristic clones were in the range of 1.6–2.3 µg/mL with an average of 2.0 µg/mL, which was comparable to the average (1.9 µg/mL) of the total 12 clones. Thus, morphological selection of parental clones can facilitate the establishment rCHO cell lines expressing TPO.

DISCUSSION

We have developed a selection method based on cell morphology,

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permitting the rapid and convenient selection of parental rCHO clones with a high expression level of TPO. Compared with the random selection of clones, selection based on morphology improved selection efficiency of clones with an expression level higher than 200 ng/mL by 3.5-fold. Furthermore, when subjected to MTX, characteristic clones displayed an elevated expression level. Thus, morphological selection of parental clones can facilitate the establishment of rCHO cell lines expressing TPO. Although cell morphometry such as the cell length/width ratio and the cell shape factor can be used for more quantitative selection (9,12), we prefer to use a conventional phase-contrast microscope for morphological selection because of its simplicity.

Morphological changes of CHO cells are known to be induced by many factors, such as attachment factors (13), cyclic AMP or the stimulation of G-protein-coupled receptors (6,9) and cul-

tivation method. When transfected, expression plasmids randomly integrate into the chromosome of host cells. Parental clones survived from transfection and drug selection stress may show various morphological changes, depending on the chromosomal integration site. Although the detailed mechanism responsible for morphological change remains to be elucidated, efficacy of morphological selection for the establishment of rCHO cell lines expressing TPO has been demonstrated.

Application of morphological selection is not limited to the establishment of rCHO clones expressing TPO and its analogs. When we transfected expression plasmids encoding neomycin-resistant marker and humanized antibody against hepatitis B virus surface antigen into dhfr CHO cells, characteristic clones were also observed, and many of them displayed high-level expression. Thus, this morphological selection will be a useful tool for selecting higher producing clones in the establishment of rCHO cell lines regardless of selection markers and expressed proteins.

In conclusion, simple morphological selection allows one to efficiently screen parental clones with high-level expression, facilitating the establishment of rCHO clones expressing recombinant proteins.

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