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A novel RNA silencing vector to improve antigen expression and stability in Chinese hamster ovary cells

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Abstract

Chinese hamster ovary (CHO) cells and dihydrofolate reductase (*dhfr*)/methotrexate (MTX) gene amplification system are routinely used to generate stable producer CHO cell clones in biopharmaceutical industries. The present study proposes a novel method by the co-amplification of the silencing vector targeted to *dhfr* gene for improvements of selecting high-producing clones in *dhfr*-deficient and wild-type CHO cells. Using the silencing vector also resulted in improving the stability of the recombinant protein expression in the absence of MTX in the CHO/dhFr$^-$ and wild-type CHO cells. This new method is proposed to generate highly expressed stable cell clones of both *dhfr*-deficient and wild-type CHO cells for recombinant antigen production. Utilization of the silencing vector designed in this study can improve antigen expression through *dhfr*-directed gene amplification in other *dhfr*-competent cell lines for vaccine development.

Keywords: RNA silencing; Dihydrofolate reductase; CHO cells; Antigen

1. Introduction

Mammalian cells have been extensively utilized to produce recombinant proteins as vaccine candidates for potential applications [1–5]. The amplifiable selective marker, such as dihydrofolate reductase (*dhfr*), and Chinese hamster ovary (CHO) cells are routinely used to generate stable producer cell clones [6,7]. Methotrexate (MTX), a folic acid analog which binds and inhibits DHFR, has been widely used to improve recombinant DNA expression in CHO cells by co-amplifying of the target gene and the *dhfr* gene concurrently [7–9]. Stepwise increasing the concentration of MTX in growth medium can result in hundred to thousand copies of the co-amplified target genes in stable producer CHO cells [7,10]. To date, several attempts have been carried out to improve the *in vitro* selection of stable producer CHO cells through stepwise MTX selection, including the internal ribosome entry site (IRES)-driven dicistronic vector [11], the incomplete splicing (in *dhfr* and target cDNA) vectors [12], and the use of less-sensitive mutant *dhfr* genes to MTX [13,14]. However, *in vitro* selection of high producer cell clones still remains as the most time-consuming step in CHO cell expression technology [15].

RNA interference (RNAi), initially found in *Caenorhabditis elegans* [16], has been considered as a natural response to double-stranded RNA for controlling sequence-specific gene expression at a post-transcriptional level [17,18]. Introducing double-stranded RNA in mammalian cells has emerged as a powerful means to silence gene expression in mammalian cells through RNAi [19–21]. The double-stranded RNAs, transcribed as short hairpin RNA (shRNA) and processed into active, 19–23 nucleotide RNAs by Dicer, can recognize the target mRNAs in a sequence-specific manner [22]. Recently, the RNAi technology has been applied to improve the recombinant DNA expression in CHO cells by silencing alpha-1,6-fucosyltransferase [23], pro-apoptotic proteins [24], and caspase-3 expression [25].

The present study proposes a novel method by the co-amplification of an expression vector and a RNA silencing vector targeted to *dhfr* gene to obtain stable producer clones in *dhfr*-deficient and wild-type CHO cells. The RNA silencing vectors were demonstrated to induce an efficient knockdown of both exogenous (mouse) and endogenous

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(Chinese hamster) *dhfr* gene expression in CHO/dhFr$^-$ and CHO-K1 cells, respectively. Combining the *dhfr*-targeted RNA silencing vector and the expression vector encoding the reporting gene of enhanced green fluorescent protein (*egfp*) yielded significant improvements for obtaining high-producing CHO cell clones with an enhanced stability in MTX-free medium. The new strategy proposed here can be applied to obtain high producer cell clones in both *dhfr* deficient and wild-type CHO cells for vaccine development.

2. Materials and methods

2.1. Construction of plasmids

Reporting vectors (pEGFP-DHFR) were generated by replacing the neomycin phosphotransferase gene (*neo*) gene with mouse *dhfr* cDNA driven by an SV40 promoter in the pcDNA3.1(+) (Invitrogen). The EGFP fragment from pEGFP-N1 (BD Biosciences), driven by cytomegalovirus immediate-early gene promoter and enhancer (CMV) was cloned into BamHI and EcoRI sites. The internal ribosomal entry site (IRES) on pIRES (BD Biosciences) and the Zeocin (*Zeo*) gene from pcDNA3.1/Zeo (Invitrogen) were cloned into XhoI/XbaI and XbaI/ApaI sites, respectively, to select colonies. The human polymerase-III U6 promoter [26] was amplified from the genomic DNA of HeLa cells (ATCC, CCL-2) and cloned in front of the mouse/hamster *dhfr*-specific shRNA with five thymidines as a terminator signal to construct shRNA silencing vectors (pCMVen-sd1, pCMVen-sd2, and pCMVen-sd3). The CMV promoter and the enhancer were replaced with a CMV enhancer upstream of the U6 promoter to increase the efficacy of silencing [27] in the identification of shRNA candidates. The control vector (pCMVen) has the same CMV enhancer/U6 promoter with no shRNA.

2.2. Cell culture and transfection

Wild-type CHO-K1 cells (CCL-61) and the *dhfr*-deficient mutant CHO/dhFr$^-$ cells (CRL-9096), were obtained from FIRDI (Taiwan) and cultured at 37 °C in a humidified incubator with 5% CO_2. CHO-K1 and CHO/dhFr$^-$ cells, before transfection and cloning selection, were maintained in Ham's F-12K and MEM-α media supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). CHO cells were transfected with expression and silencing vectors using Lipofectamine 2000 (Invitrogen).

2.3. Western blotting

CHO cells that had been transfected with expression and silencing vectors were harvested. Equal amounts of protein were resolved by SDS-PAGE (12% polyacrylamide) and transferred to a nitrocellulose membrane (Millopore) by electroblotting. Immune complexes were visualized with BCIP/NBT substrate kit (Invitrogen) using anti-DHFR IgG (BD Biosciences), anti-GAPDH IgG (Abcam) and Anti-mouse IgG-AP.

2.4. Stable clone selection and gene amplification

The CMV enhancer was removed before transfection to prevent interference by the CMV enhancer of CMV enhancer-promoter-dependent transcription [28]. The linearized plasmids (2 μg) were co-transfected into CHO-K1 and CHO/dhFr$^-$ cells with a 1:0, 1:1 or 1:5 ratio of the reporting expression vector (pEGFP-DHFR) to the psd2 silencing vector that contained only CMV promoter without the enhancer. Following transfection, the cells were transferred into two 60 mm plates, grown for two days in a nonselective growth medium, and then replaced the medium with ribonucleosite/deoxyribonucleosite-free MEM-α (Invitrogen) with 10% dialyzed serum (DS, Invitrogen), 200 μg/ml Zeocin (Invitrogen) and 600 μg/ml G-418 (Calbiochem) to select DHFR-, Neo-, and Zeo-positive colonies. Only Zeocin was used to select colonies of the control transfected cells (pEGFP-DHFR only). 10–14 days after the cells were transferred to the selective medium, a limiting dilution in the 96-well plates was employed to isolate cell clones and the wells that contained single cells were labeled under fluorescent microscopy. Single cell clones were isolated and 18 of the colonies that expressed the highest level of EGFP were employed in the subsequent methotrexate (MTX)-driven amplification. The MTX concentration in CHO cell culture medium was increased from 0.04 μM to 5 μM. In each selection step, cells were cultivated for at least 15 days before the MTX concentration was increased.

2.5. Real-time PCR and RT-PCR

The genomic DNA extraction from stable CHO cell clones was performed using DNeasy Tissue kit (Qiagen). The RNAs were extracted from the transfected cells using Trizol (Invitrogen). The primers and Taqman probe (Applied Biosystems assay ID 293340) were employed for real-time PCR measurement to determine the copy numbers of mouse *dhfr* gene. One-step RT-PCR was performed to obtain the amount of RNA transcripts of mouse and hamster *dhfr* genes. The real-time PCR and RT-PCR assays were performed on an ABI PRISM 7500 real time PCR system, by calculating the absolute amount of DNA and the relative amount of RNA by the ΔΔCt method, while the amounts of RNA transcripts were normalized to that of eukaryotic 18s rRNA.

3. Results

3.1. RNA silencing vector to dhfr gene expressed in CHO/dhFr$^-$ cells

Three target sequences (sd1, sd2, sd3) located in the conserved sequences of the mouse and Chinese hamster