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# Generation of high-producing cell lines by overexpression of cell division cycle 25 homolog A in Chinese hamster ovary cells

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**To improve the efficiency of conventional gene amplification systems, the effect of cell cycle modification during the gene amplification process on IgG production was investigated in Chinese hamster ovary (CHO) cells. The full-length cDNA of CHO cell division cycle 25 homolog A (Cdc25A) was introduced into CHO DG44 cells and the effects of CDC25A overexpression on the cell cycle, transgene copy number and IgG productivity were examined. Both wild-type and mutated CDC25A-overexpressing CHO cells showed a rapid increase in transgene copy number compared with mock cells during the gene amplification process, in both cell pools and individual clones. High-producing clones were obtained with high frequency in CDC25A-overexpressing cell pools. The specific production rate of the isolated clone CHO SD-S23 was up to 2.9-fold higher than that of mock cells in the presence of 250 nM methotrexate (MTX). Cell cycle analysis revealed that the G2 to M phase transition rate was increased ~1.5-fold in CDC25A-overexpressing CHO cells under MTX treatment. Our results show the improvement of conventional gene amplification systems via cell cycle engineering at an early stage of cell line development.**



[**Key words:** Chinese hamster ovary cells; Gene amplification; Monoclonal antibody; CDC25A; Cell cycle]

Cell cycle engineering is a useful strategy for achieving suitable production of therapeutic proteins using mammalian cell culture. Over the past two decades, researchers have primarily focused on cell cycle modulation to increase proliferation rate and viable cell density (1,2), and on cell cycle arrest to achieve productivity enhancement in Chinese hamster ovary (CHO) cells (3,4). Although significant improvements in cell growth and recombinant protein yields have been achieved by controlling the cell cycle, to our knowledge, no studies have performed cell cycle engineering to generate high-producing cells in the cell line development process using gene amplification.

To establish high-producing CHO cells, a variety of approaches have been applied in industry, with dihydrofolate reductase (DHFR)-mediated gene amplification being a popular mammalian selection system (5). Briefly, the *dhfr* gene encoding an enzyme involved in nucleotide metabolism is used as a selection marker for a gene of interest (GOI), and methotrexate (MTX) acts as a competitive inhibitor to the DHFR enzyme. High-producing cells containing the *dhfr* gene and the recombinant GOI, which are amplified by chromosome rearrangements, can be selected under MTX treatment (6,7). However, the process for the selection and screening of clones typically takes several months (8). Consequently, novel approaches are desirable to minimize time and cost in this selection and screening process. Gene amplification is caused by genomic instability, which is closely related to the cell cycle control mechanism. We focused on cell cycle engineering to enhance the efficiency of the conventional gene amplification system via cell cycle control.

In our previous study, we down-regulated a cell cycle checkpoint kinase, ataxia-telangiectasia and Rad3-related (ATR), to accelerate gene amplification (9). ATR is activated in response to DNA damaging agents and activates checkpoint kinases 1 and 2 (CHK1 and CHK2) to initiate signal cascades. In this study, we focused on the targets of these signaling pathways, cell cycle division 25 (CDC25) phosphatases. CDC25 phosphatases activate complexes of cyclins and cyclin-dependent kinases (CDKs), which in turn regulate cell cycle transitions (10). They are important targets of checkpoint machinery via CHK1 and CHK2 in response to DNA damage (11). The activities of CDC25 phosphatases must be tightly regulated to maintain precise cell cycle control. Conversely, dysregulation of their expression levels causes genomic instability (12). Among the three isoforms, CDC25A, B and C, CDC25A is considered the key component which is able to compensate for the lack of CDC25B and CDC25C (13) and is involved in both G1–S and G2–M phase transitions (14–16). Thus, overexpression of CDC25A

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leads to the bypass of DNA damage-induced checkpoint arrest, allowing cell cycle progression with the unrepaired DNA, and may finally cause chromosome instability (17–19).

In the present study, we investigated the effects of CDC25A overexpression on gene amplification and productivity during the process of productive cell line development. The changes in transgene copy number and productivity in CDC25A-overexpressing CHO cells producing a recombinant antibody were evaluated during the gene amplification process. The relationship between cell cycle transition and the frequency with which high-producing clones were obtained was also investigated.

## MATERIALS AND METHODS

**Cell culture and media** The CHO DG44 (20) cell line was used in this study. The CHO DG44 cells (dhfr $^-$) were maintained in Iscove's modified Dulbecco's medium (IMDM) (Sigma–Aldrich, St. Louis, MO, USA) with 10% dialyzed fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS, USA), 0.1 mM hypoxanthine (Yamasa, Choshi, Japan), and 0.01 mM thymidine (Yamasa). All cell lines derived from DG44 cells by transfection with vectors harboring the *Dhfr* gene were cultivated in IMDM containing 10% dialyzed FBS without hypoxanthine and thymidine at 37°C in a humidified atmosphere containing 5% $CO_2$. The cells were passaged every 3–4 days in fresh medium at a density of $1 \times 10^5$ cells/mL.

**Antibody expression plasmid and parental cell line construction** A gene encoding a humanized anti-EGFR×anti-CD3 bispecific single-chain diabody with an Fc portion (scDb-Fc) was used (21). The scDb-Fc fragment was inserted into *Nhe*I/*Xho*I-digested pcDNA 3.1-Neo (Invitrogen, Carlsbad, CA, USA). The *Dhfr* fragment was amplified by PCR from the pSV2-dhfr/hGM-CSF plasmid (22) and ligated to *Bgl*II-digested plasmid. Adherent CHO DG44 cells were transfected with the antibody expression plasmid, which was linearized by the restriction enzyme *Psp*1406I, using an X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Two days after transfection, the transfected cells were transferred to a 25-$cm^2$ T-flask and stable transfectants were selected by culturing in medium lacking H/T and containing 500 μg/mL G418 (Sigma–Aldrich, St. Louis, MO, USA). The selection medium was changed every 3 days until G418-resistant cells were obtained 2 weeks after transfection. The surviving cells (CHO-scDb-Fc) were maintained under selection pressure of 500 μg/mL G418. Individual clones were isolated from the CHO-scDb-Fc cell pool by limiting dilution. The CHO-scDb-Fc cell pool and an isolated clone, SD-S23, were used in this study.

**Construction of CDC25A expression plasmid and site-directed mutagenesis** The full-length cDNA of CHO CDC25A was cloned from a cDNA pool prepared from the mRNAs of CHO DG44 cells and fully sequenced. The sequence data have been submitted to the DDBJ database (accession number AB809654). The wild-type (wt-) CDC25A expression plasmid was constructed by insertion of the CHO CDC25A cDNA into *Kpn*I/*Bam*HI-digested pcDNA3.1/Hygro(+) expression vector (Invitrogen). To generate a double base mutant of CHO CDC25A, mutagenesis was performed by PCR using a PrimeSTAR Mutagenesis Basal Kit (Takara Bio Inc., Otsu, Japan) with the following pairs of primers: for CDC25A S76A, 5′-AATGGGCGCC TCTGAATCCACTGATT-3′ (forward) and 5′-TCAGAGGCGCCCATTCTCTGTAGACT-3′ (reverse); and for CDC25A T509A, 5′-GAGCCGGGCCTGGGCAGGGGAAAAGA-3′ (forward) and 5′-GCCCAGGCCCGGCTCTTGGTACGAAA-3′ (reverse). The mutations were confirmed by DNA sequencing. The constructed plasmids were linearized by restriction enzyme *Eam*1105I and transfected into the parental cell lines using an X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science). The stably transfected cells were selected in medium containing 500 μg/mL G418 (Sigma–Aldrich) and 500 μg/mL hygromycin (Wako, Osaka, Japan).

**Cell cycle analysis and identification of mitotic cells** Cells ($1 \times 10^6$) were harvested by centrifugation, washed with phosphate-buffered saline (PBS), fixed by adding 1 mL of 70% ethanol and stored at −20°C for at least 4 h. Fixed cells were then washed twice with PBS, suspended in 1 mL of 0.25% Triton X-100 in PBS, and incubated on ice for 15 min. After rinsing, the cell pellets were stained with 5 μL of Alexa Fluor 647 anti-Histone H3-Phosphorylated (Ser28) (BioLegend Inc., San Diego, CA, USA) in 100 μL of PBS containing 0.5% bovine serum albumin (BSA) for 30 min at room temperature. The cell pellets were rinsed with PBS, treated with 50 μg of RNase A (Sigma) in 50 μL of PBS for 30 min at 37°C, and stained with 10 μg of propidium iodide (PI) (Sigma) in 200 μL of PBS. Fluorescence was measured using a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

**Analysis of gene copy number** Quantitative real-time PCR using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) was used to determine the transgene copy number. Genomic DNA from $1 \times 10^6$ cells from each cell line was isolated using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and RNase A (Sigma–Aldrich), and 1 μL of the genomic DNA was used as a template for quantification of the transgene copy number. A Fast SYBR Green PCR Master Mix (Applied Biosystems) was used for the PCR reaction with following primers: 5′-AGGAGTACAAGTGCAAGGTCTCCAAC-3′ for IgG gene forward, 5′-ACCTGGTTCTTGGTCAGCTCATCC-3′ for IgG gene reverse. The CHO β-actin gene was used as an internal control to normalize the PCR for the amount of genomic DNA. The forward and reverse primers for the β-actin gene were: 5′-G CTGTGGGTGTAGGTACTAACAAT-3′ and 5′-GAATACACACTCCAAGGCCACTTA-3′, respectively.

The PCR reaction was performed using the following thermal cycling program: 20 s at 95°C, and 40 cycles of (3 s at 95°C and 30 s at 60°C). A series of serial dilutions for the hEx3-scDb-Fc antibody expression plasmid and β-actin fragment, which was amplified from CHO DG44 cells, was used to generate a standard curve.

**Fluorescence in situ hybridization (FISH) analysis** FISH analysis was performed as described previously (23,24). In brief, chromosome spreads were prepared using standard techniques of a 4-h colcemid treatment (20 ng/mL) followed by a 20-min incubation in 75 mM potassium chloride at room temperature, and five changes of Carnoy's fixative (3:1 methanol:acetic acid). One microgram of the hEx3-scDb-Fc expression plasmid was labeled using a biotin-nick translation mix (Roche Diagnostics, Basel, Switzerland) and hybridized with chromosome spreads. The hybridized plasmids were detected using fluorescein isothiocyanate (FITC)-labeled avidin (Fluorescein Avidin DN, Vector Laboratories, Inc., Burlingame, CA, USA). Chromosome spreads were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under an Axioskop 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

**Productivity evaluation** A standard sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of secreted antibody. Culture supernatants were added to 96-well plates coated with Fc-specific goat anti-human IgG (Bethyl Laboratories, Montgomery, TX, USA) and incubated for 2 h at room temperature. HRP-conjugated goat anti-human IgG (Bethyl Laboratories) and ABTS reaction solution (KPL, Gaithersburg, MD, USA) were used to detect captured antibody. After adding ABTS peroxidase stop solution (KPL) to the 96-well plates, absorption was measured at 405 nm using a microplate reader (Tecan, Männedorf, Switzerland). A standard curve was generated using purified immunoglobulin G from human plasma (Athens Research & Technology, Athens, GA, USA). The assay was performed in triplicate for each sample. The specific productivity was calculated based on viable cell density and the antibody concentration, as described previously (25).

**Western blot analysis** Total cellular protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) supplemented with Halt protease inhibitor cocktail (Pierce Biotechnology). The protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology). Twenty micrograms of the protein extracts was resolved by SDS-PAGE and transferred to polyvinylidine difluoride (PVDF) membranes. The membranes were incubated with primary antibody, anti-Cdc25A (M-191) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and then incubated with an HRP-conjugated anti-rabbit IgG from goat (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) as the secondary antibody. The membranes were developed with an Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore Corporation, Billerica, MA, USA) and exposed using a LAS-4000 Mini Image Reader (Fujifilm, Tokyo, Japan).

## RESULTS AND DISCUSSION

**Construction of CDC25A-overexpressing cells** To generate CHO cells overexpressing wt- or mutant CDC25A (S76A and T509A), the overexpression plasmids were transfected into the CHO-scDb-

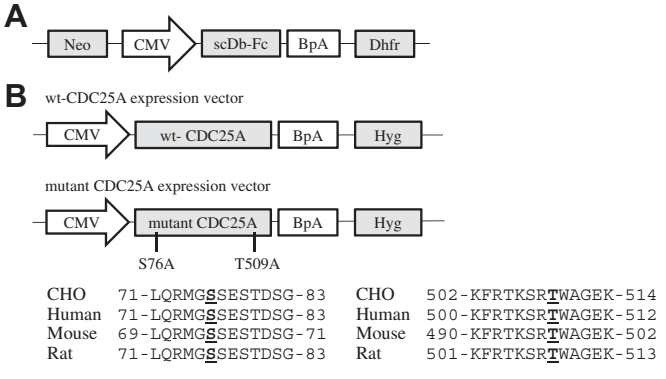


FIG. 1. Schematic representation of vectors. (A) A bispecific single-chain diabody with an Fc portion (scDb-Fc) expression vector. (B) Wt and mutant CDC25A expression vectors. Mutagenesis was performed to substitute S76 and T509 of CDC25A with alanines. Ser76 and Thr509 of CHO Cdc25A are conserved across species. CMV, human cytomegalovirus immediate-early promoter; BpA, bovine growth hormone polyadenylation signal; Neo, neomycin resistance gene; Hyg, hygromycin resistance gene.