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Increased recombinant protein production owing to expanded opportunities for vector integration in high chromosome number Chinese hamster ovary cells

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Chromosomal instability is a characteristic of Chinese hamster ovary (CHO) cells. Cultures of these cells gradually develop heterogeneity even if established from a single cell clone. We isolated cells containing different numbers of chromosomes from a CHO-DG44-based human granulocyte-macrophage colony stimulating factor (hGM-CSF)-producing cell line and found that high chromosome number cells showed higher hGM-CSF productivity. Therefore, we focused on the relationship between chromosome aneuploidy of CHO cells and high recombinant protein-producing cell lines. Distribution and stability of chromosomes were examined in CHO-DG44 cells, and two cell lines expressing different numbers of chromosomes were isolated from the original CHO-DG44 cell line to investigate the effect of aneuploid cells on recombinant protein production. Both cell lines were stably transfected with a vector that expresses immunoglobulin G3 (IgG3), and specific antibody production rates were compared. Cells containing more than 30 chromosomes had higher specific antibody production rates than those with normal chromosome number. Single cell analysis of enhanced green fluorescent protein (*Egfp*)-gene transfected cells revealed that increased GFP expression was relative to the number of gene integration sites rather than the difference in chromosome numbers or vector locations. Our results suggest that CHO cells with high numbers of chromosomes contain more sites for vector integration, a characteristic that could be advantageous in biopharmaceutical production.

[**Key words:** Chinese hamster ovary cells; Chromosomal aneuploidy; Fluorescence *in situ* hybridization; Vector integration; Recombinant protein production; Antibody production]

CHO cells have emerged as the most important host cells used for the production of biopharmaceuticals in recent years (1,2). A CHO cell line was first established from female Chinese hamster (*Cricetulus griseus*) ovary cells that could be cultured consecutively for more than 10 months with no diminution in growth rate or change in cellular or colonial morphology (3). While euploid primary Chinese hamster cells contain 22 chromosomes, cells extirpated to *ex vivo* gradually show aneuploidy (4). Genomes of several CHO cell lines, including CHO-K1, DG44, CHO-S, and DXB11, are rearranged from the original Chinese hamster genome and differ from each other (5–7). The CHO-K1 cell line, which requires proline (8), and the CHO-DG44 cell line, which requires hypoxanthine and thymidine (9), are the most commonly used CHO cell lines in the pharmaceutical industry. The CHO-DG44 cell line is a dihydrofolate reductase (DHFR)-deficient line that is frequently used as a host cell for the DHFR-mediated gene amplification method (10). Since DHFR is an enzyme involved in nucleotide synthesis, CHO-DG44 cells cannot survive without hypoxanthine and thymidine; however, exogenous *Dhfr* genes can be utilized and amplified with nearby sequences in the presence of a DHFR antagonist, methotrexate (MTX) (10). In this study, we used the CHO-DG44 cell line to evaluate the impact of chromosome aneuploidy on recombinant protein production.

Two major methods are used to transfect exogenous genes: random integration and gene targeting. To control the vector integration sites, the gene-targeting method was used in this study. The recently developed CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated nuclease 9) system (11,12) is a powerful gene-targeting method. In the conventional method, gene-targeting vectors required homologous arms for effective recombination. However, exogenous genes can be efficiently inserted into a specific region of the genome without adding homologous regions to both sides of the exogenous gene by using CRISPR-Cas9 vectors (13). A targeting sequence was obtained from the CHO genomic bacterial artificial chromosome (BAC) library (5,14) generated from the CHO DR1000L-4N cell line (15). The CHO DR1000L-4N cell line is a CHO-DG44 cell line-based recombinant cell line that produces hGM-CSF, and it was constructed via gene amplification by transfection of *Dhfr* and *CSF2* (GM-CSF gene) (15). The targeted sequence of the BAC clone identified as Cg0031N14 was found to contain an exogenous gene amplified region with a large palindromic structure (16), and this was considered an appropriate locus to insert expression vectors for recombinant protein production.

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In this study, we compared the productive capacity of cells containing different numbers of chromosomes that were isolated from the CHO-DG44-based hGM-CSF-producing cell line CHO DR1000L-4N (15). We next investigated the chromosomal variability of the CHO-DG44 cell line and compared the recombinant protein production efficiencies of cell lines constructed from two sub-clones of CHO-DG44, whose modal chromosome numbers were 20 and 39, respectively. We also investigated the reasons for differences in productivity using a gene-targeting method and clarified the relationship between the number of sites for vector integration and recombinant protein production.

MATERIALS AND METHODS

Cell culture and transfection The CHO-DR1000L-4N cell line was cultured as described previously (15) in the presence of 1000 nM MTX (Sigma–Aldrich, St. Louis, MO, USA). The CHO-DG44 cell line was provided by Dr. L. Chasin (Columbia University, New York City, NY, USA), and the cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Sigma–Aldrich) supplemented with 10 μM thymidine, 100 μM hypoxanthine, and 10% dialyzed fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA). Expression vectors were transfected using X-tremeGENE 9 DNA transfection reagent (Roche Applied Science, Mannheim, Germany). Transfected cells were cultured in IMDM containing 300 nM MTX, 10% dialyzed FBS, but without thymidine and hypoxanthine. An automated cell analyzer, Vi-cell XR (Beckman Coulter, Brea, CA, USA), was used to analyze total/viable cell concentrations and average cell diameters.

Chromosome number counting Cells in the exponential phase were used to prepare metaphase chromosome spreads as described previously (14). Chromosome spread glass slides were stained with 200 ng/mL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min. Chromosome numbers were counted under an Axioskop 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Kinetic parameters The specific growth and production rates were calculated as follows (17). Cells were seeded at the density of 1.0×10^5 cells/well in 6-well plates, and samples of viable cells and cell supernatants were obtained 24, 48, 72, 96, 120 and 144 h after the initial seeding. Recombinant protein concentrations of the cell supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (18,19). Briefly, biotinylated mouse anti-human GM-CSF (R&D Systems, Minneapolis, MN, USA) or goat anti-human IgG-Fc (Bethyl Laboratories, Montgomery, TX, USA) was used as a capture antibody, and streptavidin-horseradish peroxidase (HRP) (R&D Systems) or HRP-conjugated goat anti-human IgG (Bethyl Laboratories) was used as a detection antibody. *Escherichia coli* recombinant human GM-CSF (R&D Systems) or IgG3 kappa from human myeloma plasma (Sigma–Aldrich) was used as a reference protein.

Analyses of vector integration sites Dual-color or single-color fluorescence *in situ* hybridization (FISH) analysis was performed as described previously (5) to identify the number and locations of vector integration sites. In case of dual-color FISH analysis, expression vectors were labeled with biotin, and BAC clone DNA was labeled with digoxigenin for use as a hybridization probe. Specifically, the Cg0031N14 BAC clone (16) was used to identify the targeted location of CRISPR/Cas9. Vector integrations into the targeted location were determined by overlapped signals that detect expression vector probes and the Cg0031N14 BAC probes.

Antibody expression plasmid The Mammalian PowerExpress System (Toyobo, Osaka, Japan) was used to construct an IgG3 expression vector. The Ig gamma-3 chain, internal ribosome entry sites (IRES), and *Dhfr* (F31S) (20) fragments were ligated into the pEHX1.1 vector. The Ig kappa chain fragment was ligated into the pELX2.1 vector. Finally, the heavy chain expressing the pEHX1.1 vector and the light chain expressing the pELX2.1 vector were integrated into one plasmid. The Ig gamma-3 chain consists of heavy chain variable region (prepared in-house) and human Ig gamma-3 chain C region (UniProt ID: P01860), and Ig kappa chain consists of light chain variable region (prepared in-house) and human Ig kappa chain C region (UniProt ID: P01834). The gene products of Ig gamma-3 chain and Ig kappa chain were synthesized (Tosoh, Tokyo, Japan).

GFP expression plasmid *Egfp* and *Dhfr* fragments were ligated into the pcDNA3.1(+) vector (Life Technologies, Carlsbad, CA, USA).

Gene targeting The CAG-hspCas9-H1-guide RNA scaffold SmartNuclease All-in-one vector (System Biosciences, Mountain View, CA, USA) was used. A vector that expresses a guide RNA sequence (GAGTGGAAAATAGATGGCCT) that targets a sequence in the CHO genomic BAC library Cg0031N14 clone (16) was constructed. Gene-targeting vectors without homology arms were co-transfected with the CRISPR/Cas9 vector.

Measurement of GFP expression in cells containing each vector integration pattern Single cell clones were obtained from the GFP expression vector-transfected cell pools. GFP expression over a threshold intensity was analyzed by BD FACSVerse Flow Cytometer (BD Biosciences, San Jose, CA, USA). Cells were categorized according to the numbers and sites of vector integration, and GFP expression was compared between these groups.

RESULTS

Isolation of cells containing different numbers of chromosomes from the hGM-CSF-producing cell line Sub-clones of cells containing different numbers of chromosomes were isolated from the hGM-CSF producing CHO-DG44-based CHO DR1000L-4N cell line. A cell with 18 chromosomes was isolated and identified as sub-clone (SC) 18, and correspondingly, SC19, SC20, and SC30+ (over 30 chromosomes) were isolated and named. While the diameters of SC20 cells were consistently measured to be 14–16 μm, the diameters of SC30+ cells were measured to be 16–19 μm (Fig. 1A). Interestingly, the specific protein production rate of SC30+ cells was higher than that observed for the other clones (Fig. 1B), and the chromosomes that contained an exogenous gene-amplified region were doubled in SC30+ cells (Fig. 1C). To investigate the productive capacity of cells containing high chromosome numbers more thoroughly, we isolated cells containing different numbers of chromosomes from the non-producing CHO-DG44 cell line, and recombinant protein-producing cells derived from these were assessed for productivity. Chromosome number heterogeneity of the CHO-DG44 cell line was examined prior to productivity analysis.

CHO-DG44 chromosome number heterogeneity Distribution of the number of chromosomes in the CHO-DG44 cell pools was determined by DAPI-stained metaphase spreads. Most cells contained approximately 21 chromosomes, while 4% of the cells had more than 30 chromosomes (Fig. 2A). The distributions of the numbers of chromosomes within the sub-clones isolated from the CHO-DG44 cell line were traced for 3 months of continuous culture. Although the number of chromosomes tended to change more easily in SC30+ cells compared to SC20, SC21, or SC22 cells (Fig. 2B and C), SC30+ cells maintained their high chromosome number for at least 3 months (Fig. 2C).

Establishment of antibody-producing cell lines with different numbers of chromosomes Two sub-clones, designated DG44-SC20 and DG44-SC39, whose modal chromosome numbers were 20 and 39, respectively, were isolated from parental CHO-DG44 cells (Fig. 3). The diameters of DG44-SC20 cells were measured as 13.41 ± 2.32 μm ($n = 196$), and those of DG44-SC39 cells were measured as 17.55 ± 4.07 μm ($n = 73$). To construct antibody-producing cell lines, DG44-SC20 and DG44-SC39 were stably transfected via random integration method with an expression vector containing the *IgG3* and *Dhfr* genes, following which IgG3-SC20 and IgG3-SC39 cell lines were established. Gene amplification in the presence of 300 nM MTX was performed following transfection. The diameters of IgG3-SC20 cells were measured as 15.35 ± 3.09 μm ($n = 110$), and those of IgG3-SC39 cells were measured as 17.93 ± 3.03 μm ($n = 79$). No difference in specific growth rate was observed between IgG3-SC20 and IgG3-SC39 (Table 1).

Antibody production in cells with different numbers of chromosomes The rate of specific protein production by IgG3-SC39 without gene amplification was approximately eight times higher than that of IgG3-SC20 (Table 1). Correspondingly, the specific production rate of IgG3-SC39 with gene amplification in the presence of 300 nM MTX was approximately five times higher than that of IgG3-SC20 (Table 1). Evaluation of antibody titer following culture of single cell clones obtained from limiting dilution in 96-well plates demonstrated a higher antibody concentration in IgG3-SC39 compared to IgG3-SC20 with or