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Lengthening of high-yield production levels of monoclonal antibody-producing Chinese hamster ovary cells by downregulation of breast cancer 1

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The establishment process of high-producing Chinese hamster ovary (CHO) cells for therapeutic protein production is usually laborious and time consuming because of the low probability of obtaining stable, high-producing clones over a long term. Thus, development of an efficient approach is required to establish stable, high-producing cells. This study presents a novel method that can efficiently establish sustainably high-producing cell lines by acceleration of transgene amplification and suppression of transgene silencing. The effects of breast cancer 1 (BRCA1) downregulation on gene amplification efficiency and long-term productivity were investigated in CHO cells. Small interfering RNA expression vectors against BRCA1 were transfected into the CHO DG44-derived antibody-producing cell clone. Individual cell clones were obtained after induction of gene amplification in the presence of 400 nM methotrexate, which were cultured until passage 20. BRCA1-downregulated cell clones CHO B1Sa and B1Sb displayed 2.2- and 1.6-fold higher specific production rates than the S-Mock clone. Fluorescence *in situ* hybridization showed that transgene amplification occurred at a high frequency in B1Sa and B1Sb clones. Moreover, B1Sa and B1Sb clones at 20 passages had approximately 3.5- and 5.3-fold higher productivity than the S-Mock clone. Histone modification analysis revealed a decrease in an active mark for transcription, trimethylation of histone H3 at lysine 4 (H3K4), in the transgene locus of the S-Mock clone. However, H3K4 trimethylation levels were not decreased in B1Sa and B1Sb clones during long term culture. Our results suggest that high-producing cells, which maintain their productivity long-term, were efficiently established by BRCA1 downregulation.

[**Key words:** Chinese hamster ovary cells; Breast cancer 1; siRNA; Cell cycle checkpoint; Gene amplification; Histone modification; Chromatin remodeling; Recombinant protein production; Monoclonal antibody]

Chinese hamster ovary (CHO) cells are the most commonly used mammalian host cell in the biopharmaceutical industry to produce monoclonal antibodies. During production, even when the same expression vector is introduced into the same parental host cell line, the expression levels of the transgene differ between each cell and, frequently, the transgene is not expressed at all or at low levels (1). Therefore, it is generally laborious and time-consuming to obtain a high-producing recombinant cell clone from the transfected cell population, because large-scale cell screening is required to isolate several high-producing clones. Although much of the time and labor required for the screening process of high-producing clones can be reduced by modern technologies and robotics, it still requires many months and effort to acquire high-producing cells. High-producing CHO cells have been generally established using the dihydrofolate reductase (DHFR)-mediated gene amplification system (2,3). Briefly, cell clones in which target genes have been amplified along with *dhfr* can be selected in the presence of methotrexate (MTX) that competitively inhibits DHFR (4,5). Although this gene amplification system has been widely used, the detailed mechanism of gene amplification remains to be elucidated, and methods to effectively induce gene amplification have not become well established. Even after stepwise gene amplification by increasing the MTX concentration, time is still required for large scale screening to obtain clones with a high copy number of the transgene (6,7). Therefore, a more efficient method to accelerate the transgene amplification process by increasing the frequency of amplification has been desired to reduce the time required for establishment of high-producing cell lines. In our previous studies, we focused on cell cycle checkpoint regulation to enhance the efficiency of gene amplification (8–11). It has been strongly suggested that gene amplification is caused by the breakage-fusion-bridge cycle triggered by DNA double-strand breaks (DSBs) (5,12). DNA damage such as DSBs is detected by cell cycle checkpoints and then repaired. Therefore, we hypothesized that strict cell cycle checkpoints reduce the gene amplification efficiency. Furthermore, it appears that an increase of cells with unrepaired DNA damage that compulsively escapes from cell cycle checkpoint controls may accelerate the frequency of gene amplification. To test the hypothesis that weakened cell cycle checkpoints accelerate gene amplification, we performed (i) downregulation of ataxia telangiectasia and rad3-related (ATR) (8), (ii) overexpression of cell cycle division 25A (CDC25A) and mutant CDC25A (9), and (iii) overexpression of mutant CDC25B (10,11) in recombinant

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protein-producing cells. As a result, all of these engineered cell lines had increased transgene amplification efficiency and recombinant protein productivity, which strongly suggested that cell cycle checkpoint control accelerates gene amplification and generates high-producing cells. In the mutant CDC25B experiment, we attempted to change cell cycle checkpoint regulation only during cell line development, because constant weakened cell cycle checkpoints are thought to cause genomic instability. The results of this study indicated that conditional regulation of cell cycle checkpoints using the Cre-*loxP* system enhances gene amplification efficiency and maintains chromosomal stability in the established cell lines, but their specific production rates decrease significantly after repeated subculture (11).

Decreased productivity in long term cultures is a profound problem because it seriously impairs time and cost efficiency. Therefore, it is important to focus not only on efficient establishment of high-producing cell lines but also the continuity of transgene expression. Several studies have reported that the expression patterns in high-producing cell clones often become unstable and result in decreased transgene expression during long term culture. Notably, some studies have reported production instability of recombinant proteins in long term cultivation when general mammalian expression systems, including DHFR/MTX and GS/MSX gene amplification systems, are used to establish high-producing mammalian cell lines (13–17). One of the main reasons for production instability has been reported to be epigenetic silencing of transgenes. For example, histone modifications and DNA methylation at the vicinity of transgenes result in repressive chromatin structure formations (18–21). Indeed, it has been reported that unfavorable epigenetic alterations, such as aberrant DNA methylation and histone H3 hypoacetylation, exacerbate productivity losses of productive CHO cells (20–23). These types of gene silencing can be triggered by the presence of multiple transgene copies (24–27). Therefore, high-producing cells with high transgene copy numbers, particularly cell lines established by the gene amplification system, are highly susceptible to transgene silencing. To relieve chromatin-dependent gene silencing, several methods have been attempted, such as the addition of histone deacetylase and DNA methylation inhibitors (20,28) or the use of foreign genes containing vectors including specific DNA elements such as STAR (stabilizing anti-repressor), MAR (matrix attachment region), and UCOE (ubiquitous chromatin opening elements) (29–31). However, to our knowledge, there is no previous report of a method that improves the cell character itself in a way that suppresses loss of recombinant protein production caused by gene silencing in CHO cells.

In this study, we focused on breast cancer 1 (BRCA1), which operates in both cell cycle checkpoints and chromatin remodeling, to effectively establish cell lines that can maintain their high productivity for a long period of time. Direct and indirect interactions of BRCA1 with numerous proteins are known to be involved in many important biological processes such as DSB repair, S and G2/M phase cell cycle checkpoints, transcriptional regulation of several genes, and control of centrosome numbers (32–38). In addition, recent studies have shown that BRCA1 participates in heterochromatin maintenance and epigenetic mechanisms such as DNA methylation and microRNA biogenesis (39–42). Some studies have reported that BRCA1 mutations disrupt cell cycle checkpoints and allow DNA-damaged cells to progress into mitosis (33,43,44). BRCA1 deficiency also affects several DNA epigenetic modifications and leads to an open chromatin configuration that disrupts gene silencing at the tandemly repeated DNA region (39,40).

We examined whether it is possible to establish stable, high-producing cell lines over the long term by (i) breaking cell cycle checkpoints to enhance the efficiency of transgene gene amplification, and (ii) inhibiting heterochromatinization to suppress transgene silencing by BRCA1 downregulation (Fig. 1). We investigated the influences of BRCA1 downregulation in CHO cells by expressing small interfering RNA (siRNA) on gene amplification frequency and the stabilities of chromosomes and productivity during long term culture.

MATERIALS AND METHODS

Cell culture CHO DG44 (*dhfr*-) cells (45) were cultured in Iscove's modified Dulbecco's medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% dialyzed fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), 0.1 mM hypoxanthine (Yamasa, Chiba, Japan), and 0.01 mM thymidine (Yamasa). DG44-derived cell lines transfected with a plasmid containing the *dhfr* gene were cultivated in medium without hypoxanthine or thymidine. They were cultured at 37 °C with 5% CO_2.

Construction of the antibody expression plasmid and the parental cell line The light chain (LC) of human IgG1 was inserted into *Eco*RI and *Nhe*I-digested pELX2.1 (Toyobo, Osaka, Japan) (pELC), and the heavy chain (HC) of human IgG1 was inserted into *Eco*RI and *Nhe*I-digested pEHX1.1 (Toyobo) (pEHC). The

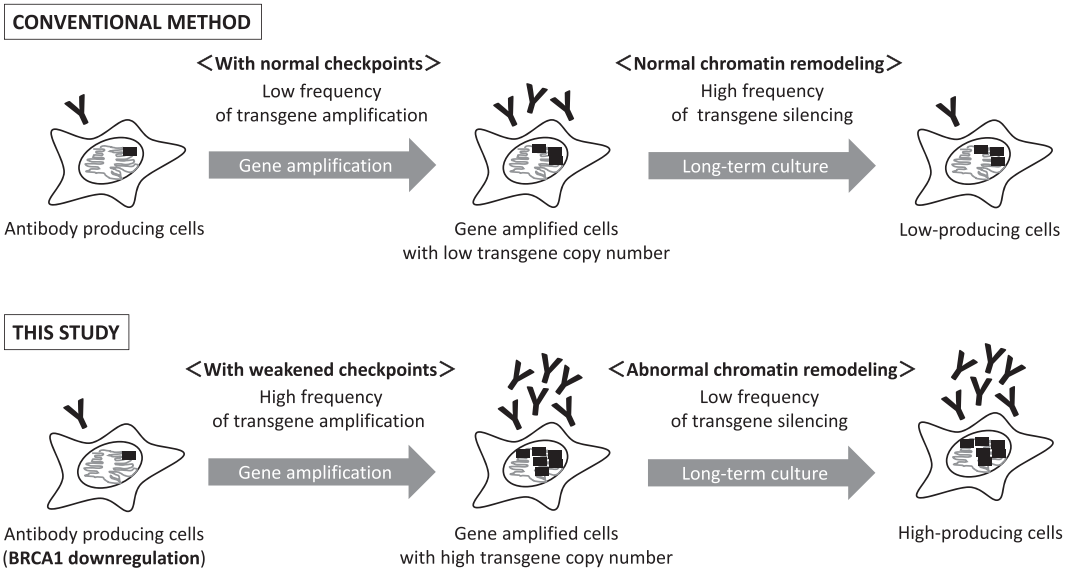

FIG. 1. Overview of stable, high-producing cell line establishment by breast cancer 1 (BRCA1) downregulation.

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