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Rapid construction of transgene-amplified CHO cell lines by cell cycle checkpoint engineering

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Abstract The process of establishing high-producing cell lines for the manufacture of therapeutic proteins is usually both time-consuming and laborious due to the low probability of obtaining high-producing clones from a pool of transfected cells and slow cell growth under the strong selective pressure of screening to identify high-producing clones. We present a novel method to rapidly generate more high-producing cells by accelerating transgene amplification. A small interfering RNA (siRNA) expression vector against ataxia telangiectasia and Rad3 related (ATR), a cell cycle checkpoint kinase, was transfected into Chinese hamster ovary (CHO) cells. The influences of ATR downregulation on gene amplification and the productivity were investigated in CHO cells producing green fluorescent protein (GFP) and secreting monoclonal antibody (mAb). The ATR-downregulated cells showed up to a 6-fold higher ratio of GFP-positive cells than that of the control cell pool. Moreover, the downregulated mAb-producing cells had about a 4-fold higher specific production rate and a 3-fold higher volumetric productivity as compared with the mock cells. ATR-downregulated cells showed a much faster increase in transgene copy numbers during the gene amplification process via methotrexate (MTX) treatment in both GFP- and mAb-producing cells. Our results suggest that a pool of high-producing cells can be more rapidly generated by ATR downregulation as compared with conventional gene amplification by MTX treatment. This novel method may be a promising approach to reduce time and labor in the process of cell line development.

Keywords Chinese hamster ovary (CHO) cells · Gene amplification · Monoclonal antibody · ATR

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Introduction

Dihydrofolate reductase (DHFR)-mediated gene amplification has been widely used to establish high-producing mammalian cell lines (Gandor et al. 1995; Kim et al. 2012; Wurm 2004). Briefly, *dhfr* gene encoding the DHFR enzyme is used as a selectable marker and methotrexate (MTX) acts as a competitive inhibitor to the DHFR enzyme. Clones containing the amplified *dhfr* gene and a gene of interest (GOI) can be selected together under MTX treatment (Kaufman 1990; Omasa 2002). However, since gene amplification is an infrequent event, in that many rounds of MTX selection to amplify the transgene and screening of over several hundred individual clones are required to obtain cells with high gene copy numbers (Cacciatore et al. 2010). Consequently, the process for DHFR-mediated gene amplification is a time-consuming and laborious step for cell line construction.

In order to enhance the selection efficiency in a DHFR-mediated amplification system, several modified methods, such as (1) dual marker amplification (Peroni et al. 2002), (2) destabilization sequences on *dhfr* markers (Ng et al. 2007), and (3) DHFR enzyme expression via incomplete splicing (Xiong et al. 2005), have been developed. However, these methods primarily focus on the stringent selection of clones having high copy numbers of GOI and/or transcripts in a pool of cells. Thus, the step to generate high-producing cells in a

heterogeneous pool, usually achieved by stepwise increases of MTX concentration (Yoshikawa et al. 2000a; Yoshikawa et al. 2000b), remains rate limiting for cell line construction. Recently, a novel approach in cell engineering for improvement of cell growth and metabolism of *dhfr*-deficient Chinese hamster ovary (CHO) cell was reported (Jeon et al. 2011). However, it would still be highly desirable to develop a more efficient method which could accelerate the transgene amplification process by rapidly increasing the frequency of amplification in a pool of heterogeneous cells.

Here, we present a novel concept to accelerate gene amplification through cell cycle checkpoint engineering. Ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are cell cycle checkpoint kinases that act as master regulators of DNA damage response by signaling to control cell cycle transitions, DNA replication, DNA repair, and apoptosis (Cimprich and Cortez 2008). Since the crucial role of ATR is to maintain genome integrity, the frequency of fragile site expression, which exhibits gaps or breaks on metaphase chromosomes, is strongly increased in ATR-deficient cells (Casper et al. 2002; Glover et al. 1984; Glover et al. 1986). In the classical breakage–fusion–bridge (BFB) model, fragile site expression plays an important role to induce gene amplification as the initial event, which is followed by sister chromatid fusion and bridge formation (Coquelle et al. 1997; Ma et al. 1993; Martins et al. 1993). Thus, we hypothesize that the increase of fragile site expression on metaphase chromosomes by forcing cells to escape from cell cycle checkpoint control under MTX treatment may accelerate the frequency of gene amplification. Figure 1 shows the concept of cell cycle checkpoint engineering. Stepwise increases of MTX concentration cause DNA replication stress in the *S*-phase of CHO cells. In general, the cell cycle checkpoint regulator causes cell cycle arrest under DNA replication stress (Casper et al. 2002). Most of the arrested cells cannot progress with DNA replication due to nucleotide imbalance and are removed by apoptosis. A few cells could escape the cell cycle check and re-enter the M-phase. These strict cell cycle checks cause cell cycle arrest and reduce gene amplification efficiency. Cell cycle checkpoint engineering controls the activity of the major checkpoint regulator ATR, accelerates cell cycle progression, and achieves quick adaptation under the increased MTX concentration.

To our knowledge, no previous report focused on controlling the cell cycle checkpoint to enhance the efficiency of the DHFR gene amplification system. Roobol et al. (2011) reported that ATR is activated by mild hypothermia in CHO-K1 cells. In this study, we investigated the influences of ATR downregulation by small interfering RNA (siRNA) on gene amplification and productivity. The change of transgene copy number and productivity in CHO cells producing green fluorescent protein (GFP) and monoclonal antibody (mAb) was analyzed in a pool of cells during the gene amplification process.

Materials and methods

Cell lines and culture

The CHO DG44 (dhfr$^-$) (Urlaub and Chasin 1980) cell line was used in this study and maintained in Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich, St. Louis, MO) with 10 % dialyzed fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS), 13.6 mg/L hypoxanthine

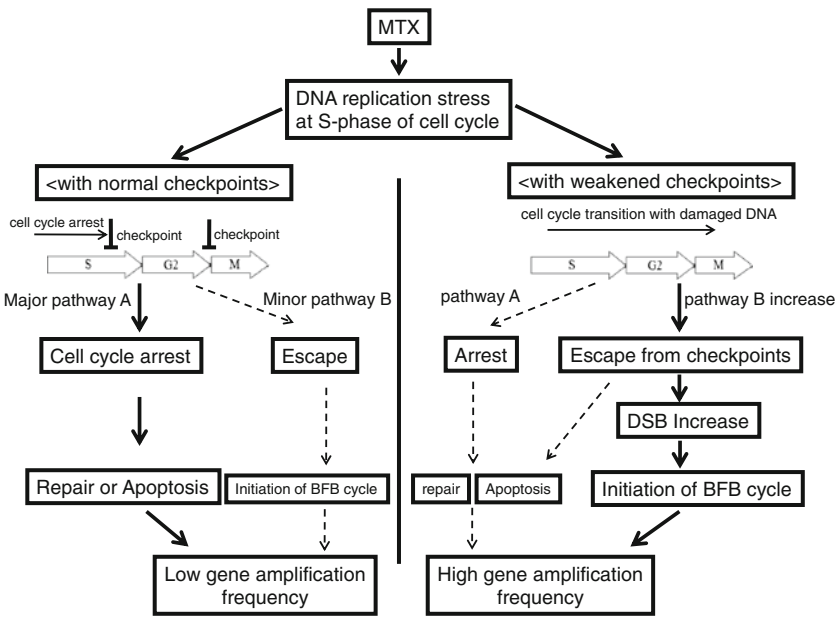

Fig. 1 Model of the relationship between cell cycle checkpoints and gene amplification frequency. *DSB* double-strand break, *BFB* breakage–fusion–bridge

(Yamasa, Choshi, Japan), and 2.42 mg/L thymidine (Yamasa). All subclones constructed from DG44 cells by transfection of vectors harboring the *DHFR* gene were cultured in IMDM containing 10 % dialyzed FBS without hypoxanthine and thymidine at 37 °C in a humidified atmosphere containing 5 % CO_2. In this study, in order to eliminate the effects of serum-free medium on the cell line construction, the simple serum containing medium was used during the MTX-adaptation process. The transfected cells were cultured under MTX (Sigma-Aldrich) at various concentrations for further experiments. The cells were passaged every 3 to 4 days in fresh medium at a density of 1×10^5 cells/mL.

Construction of ATR siRNA expression plasmid

The full-length cDNA of CHO ATR was cloned from a cDNA pool prepared from the mRNAs of CHO K1 cells and sequenced. The sequence data have been submitted to the DDBJ database (accession number AB761036). The target site for ATR gene silencing, 5′-GGAATTCTGTTGCTGAGA A-3′, was selected by RNAi Designer (Invitrogen, Carlsbad, CA) and the hairpin sequence, 5′-TTCAAGAGA-3′, was chosen from OligoEngine's protocol (OligoEngine, Seattle, WA). The oligonucleotide was obtained from Gene Design Inc. (Ibaraki, Japan) and the double-stranded oligonucleotide was ligated into the *Bam*HI/*Hin*dIII site of the pBAsi-hH1-puro expression vector (Takara Bio Inc., Otsu, Japan). The transcript from the siRNA expression vector is predicted to form a stem-loop structure and produce a functional siRNA in cells (Brummelkamp et al. 2002).

Construction of recombinant protein expression plasmids

The GFP expression plasmid, pSV2-dhfr/GFP, was described in our previous study (Park et al. 2010). Briefly, GFP and Zeocin fragments, which were obtained by PCR reaction from EGFP (Clontech, Mountain View, CA) and pZeoSV2(+) plasmids (Invitrogen), respectively, were ligated into the pSV2-dhfr/hGM-CSF vector (Yoshikawa et al. 2000a) after removal of the *hGM-CSF* gene.

The IgG expression vectors, pcDNA 3.1-Neo-HC and pcDNA 3.1-Hyg-DHFR-LC, were constructed for further study. Heavy- and light-chain genes of humanized IgG were kindly provided by Prof. Yoshikazu Kurosawa of Fujita Health University. The heavy-chain fragment was inserted into *Hind*III/*Eco*RI-digested pcDNA 3.1-Neo (Invitrogen). The light-chain expression plasmid was constructed by insertion of the light-chain fragment into *Hin*dIII/*Eco*RI-digested pcDNA 3.1-Hyg (Invitrogen). The *Dhfr* fragment was amplified by PCR from pSV2-dhfr/hGM-CSF and added to *Mlu*I/*Nru*I-digested pcDNA 3.1-Hyg-LC as a selective marker.

Cell line construction

Adherent CHO DG44 cells grown in six-well plates in IMDM (Sigma-Aldrich) supplemented with 10 % FBS (SAFC Biosciences), 13.6 mg/L hypoxanthine (Yamasa), and 2.42 mg/L thymidine (Yamasa) were transfected with the ATR siRNA expression plasmid using TransIT-LT1 reagent (Mirus, Madison, WI) according to the manufacturer's instructions. After 2 days of transfection, the transfected cells were transferred to a 25-cm^2 T-flask and the culture medium was replaced with a selection medium containing 15 μg/mL of puromycin (Wako, Osaka, Japan) to select cells harboring ATR siRNA expression plasmids. This selection medium was changed every 3 days until puromycin-resistant clones were obtained after 2 weeks of transfection. The surviving cells (CHO-siATR) were maintained under a selection pressure of 15 μg/mL puromycin. A mock cell line (CHO-mock) was generated by transfection of null pBAsi-hH1 puro expression vector into CHO DG44 cells according to the same protocol that was described above. For further study, a GFP expression plasmid was super-transfected into CHO-siATR and CHO-mock cells. Prior to the transfection, the plasmid was linearized by restriction enzyme *Apa*I. Transfection was performed according to the same protocol that was described above. The super-transfected cells were cultured in IMDM containing 50 nM MTX without hypoxanthine and thymidine for 2 weeks. IgG heavy- and light-chain plasmids were also super-transfected into CHO-siATR and CHO-mock ATR cells. Selection was performed using 50 nM MTX for the light-chain plasmid and 500 μg/mL G418 for the heavy-chain plasmid for 2 weeks. The GFP-producing super-transfected cells were cultured at 100, 250, and 500 nM MTX to evaluate gene amplification. The IgG-producing super-transfected cells were also cultured at 100 nM MTX. The obtained MTX-resistant cell pools were analyzed for further evaluation.

Analysis of ATR expression level by real-time PCR

Total RNA was isolated from CHO-siATR and CHO-mock cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with a recombinant DNase I (Takara Bio Inc.) and a RNase inhibitor (Takara Bio Inc.) for DNA removal. RNA purity was assessed using A_{260}/A_{280} ratio of samples. RNA samples having A_{260}/A_{280} ratio between 1.8 and 2 were used for the analysis. Quantification of ATR mRNA was performed using the SYBR Green quantitative real-time PCR method (Applied Biosystems, Foster City, CA), as described previously (Ohya et al. 2008). The CHO β-actin gene was used as an internal standard. The primers used for the real-time PCR were 5′-TTCTCCACAGGCACAGTCAC-3′ (forward) and 5′-GCTCATCAGGGCCCAATTAT-3′ (reverse) for the CHO ATR gene and 5′-ACTCCTACGTGGGTGACGAG-3′ (forward) and 5′-AGGTGTGGTGCCAGATCTTC-3′

(reverse) for the CHO β-actin gene. The two-step RT-PCR method was used for the analysis and PCR reaction was performed with the following thermal program: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative standard curves were generated using diluted control samples for the quantification of mRNA.

Analysis of chromosomal gaps and breaks

Chromosome spreads were prepared from cells in the exponential growth phase using standard techniques of a 4-h colcemid treatment (20 ng/mL) followed by 20-min incubation in 75 mM KCl at 37 °C and five changes of Carnoy's fixative (3:1 methanol/acetic acid ratio) (Cao et al. 2012a; Cao et al. 2012b). Cells were dropped onto slides and dried with a metaphase spreader (Hanabi, ADSTEC, Funabashi, Japan). Chromosome spreads were stained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under an Axioskop 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Photographs were taken with a CCD camera AxioCam MRm (Carl Zeiss). Metaphase spreads were scored for gaps and breaks by direct microscopic examination (Musio et al. 2005).

Flow cytometry analysis

The ratio of GFP-positive cells was analyzed by flow cytometry as described in our previous study (Park et al. 2010). Fluorescence was measured with a Beckman Coulter EPICS-XL-MCL flow cytometer (Beckman Coulter, Brea, CA) equipped with a 15-mW air-cooled argon-ion laser-emitting light at 488 nm. Cells were cultured in IMDM with 10 % dialyzed FBS at various MTX concentrations until they reached a confluent state. After removal of the media, the cells were washed with phosphate-buffered saline (PBS) followed by treatment with 0.025 % trypsin for 5 min at 37 °C and collected by centrifugation at 100×*g*. The cell pellets were resuspended in PBS at a density of 1×10^6 cells/mL. Acquisition and analysis of 5,000 events were performed using Coulter XL system II software.

Analysis of copy number of objective gene

The transgene copy number was determined by quantitative real-time PCR using a StepOnePlus™ Real-Time PCR system (Applied Biosystems). Genomic DNA from 1×10^6 cells from each cell line was isolated using a DNeasy Blood and Tissue Kit (Qiagen) and RNase A (Sigma-Aldrich), and 2 μL of the genomic DNA was directly used as a template in the real-time PCR for absolute quantification of the transgene copy number. The PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) and following primers: 5′-CACATGAAGCAGCACGACTT-3′ for GFP gene forward, 5′-GGTCTTGTAGTTGCCGTCGT-3′ for GFP gene reverse. The CHO β-actin gene was used as an internal control to normalize the amount of gDNA. The forward and reverse primers for the β-actin gene were 5′-GCTG TGGGTGTAGGTACTAACAAT-3′ and 5′-GAATA CACACTCCAAGGCCACTTA-3′, respectively.

The PCR reaction was performed with the following thermal program: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Serial dilutions for the GFP expression plasmid, pSV2-dhfr/GFP, and β-actin fragments which were amplified from CHO DG44 cells, were used to generate standard curves. A plot of C_T values versus the logarithm of the known gene copy number was constructed as a standard curve using regression analysis. The transgene copy number of each sample was calculated by relating the measured C_T values to the standard curve. The same protocol was used for the transgene copy number for IgG-producing cell lines using the following primers:

5′-GCGTGAGGAGCAGTACAACA-3′ for IgG heavy-chain gene forward,
5′-TGTTGGAGACCTTGCACTTG-3′ for IgG heavy-chain gene reverse,
5′-GTACCAGCAGAAGCCAGGAC-3′ for IgG light-chain gene forward, and
5′-TGATGGTCAAGGAAGCTGTG-3′ for IgG light-chain gene reverse.

Evaluation of specific productivity

The concentrations of IgG in the culture supernatants were measured by using a standard sandwich enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated overnight with Fc-specific goat anti-human IgG (Bethyl Laboratories, Montgomery, TX) before blocking for 1 h with 1 % BSA in PBS. Diluted culture supernatants were added to individual wells and incubated for 2 h at room temperature. Captured IgG was detected with HRP-conjugated goat anti-human IgG (Bethyl Laboratories) using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reaction solution (KPL, Gaithersburg, MD). The reaction was stopped by adding 100 μL/well of ABTS peroxidase stop solution (KPL). Absorption was measured at 405 nm using a microplate reader (Tecan, Männedorf, Switzerland). Purified immunoglobulin G from human plasma (Athens Research & Technology, Athens, GA) was used as a standard. The specific IgG production rate was calculated from the IgG concentration and viable cell density, as described previously (Kim et al. 2010; Omasa et al. 1992; Onitsuka et al. 2012). For each sample, the assay was performed in triplicate.

Results

Downregulation of ATR by siRNA

To generate cell pools having the long-term effect of ATR knockdown, siRNA (anti-ATR) expression plasmids allowing continual production of siRNAs were transfected into adherent CHO DG44 cells. Null plasmids were also transfected into CHO DG44 cells to generate a control cell line. The transfected cells were cultured under 15 μg/mL puromycin pressure for 2 weeks, and two cell pools, CHO-siATR and CHO-mock, were generated. ATR mRNA expression levels in both cell pools were investigated by real-time PCR to confirm ATR downregulation. As shown in Fig. 2, ATR gene expression in the pool of CHO-siATR cells was reduced to an average of 29 % as compared with the control cells, CHO-mock cells. In order to confirm whether siRNA expression plasmids could stably downregulate ATR expression in CHO-siATR cells for over 2 months, ATR mRNA expression levels were analyzed in both CHO-siATR and CHO-mock cells after about 70 days of cell passaging. ATR gene expression in CHO-siATR cells was constitutively reduced to an average of 38 % as compared with the mock cells. The results showed that the siRNA expression plasmid had successfully downregulated ATR gene expression to around the 30 to 40 % level as compared with the mock cells, and the over 2-month effect of the siRNA expression plasmid was enough to show that the effect was sustained.

Analysis of GFP expression level during gene amplification process

To investigate the effect of ATR downregulation on recombinant protein production, GFP-producing cell lines were generated by transfection of GFP expression plasmids into CHO-siATR and CHO-mock cells. The ratio of GFP-expressing cells in the pools of both cell lines was evaluated by flow cytometry analysis during the gene amplification process at 100-, 250-, and 500-nM MTX concentrations. All samples for flow cytometry were prepared before the first passage of MTX-treated cells in a confluent state. CHO DG44 cells were used as a negative control to set a standard value in flow cytometry (data not shown). In the process of gene amplification at all MTX concentrations, the pools of ATR-downregulated cells showed a much higher percentage of GFP-positive cells as compared with the pools of mock cells (Fig. 3). At 100-nM MTX concentration, the percentage of GFP-positive cells in the CHO-siATR cell pool was 18.7 % of total cells, which was approximately twice of the 8.4 % in the mock cells. At 250- and 500-nM MTX concentrations, CHO-siATR cell pools had 28.6 and 39.2 % GFP-positive cells, respectively, which were up to six times higher than the 4.6 and 6.8 % of the pools of mock cells. Importantly, as shown in Fig. 3, ATR-downregulated cells always showed a high ratio of GFP-positive cells at all MTX concentrations, suggesting that much higher GFP-producing cells were generated in the pool of ATR-downregulated cells as compared with that of the mock cells. Moreover, the ratios of GFP-positive cells were proportionally increased in the pools of ATR-downregulated cells with MTX concentration. However, those in the mock pools were not proportional to MTX concentration, showing even lower positive cell ratios at high MTX concentrations (250 and 500 nM) than that of the 100-nM MTX concentration. This phenomenon was confirmed by fluorescence microscopic observation of ATR-downregulated cell pools, consistent with the results of flow cytometry analysis (data not shown).

Analysis of chromosomal aberrations in ATR-downregulated cell pools

Metaphase chromosomes of CHO-siATR and CHO-mock cells producing GFP proteins were prepared by colcemid treatment. Metaphase spreads were analyzed by DAPI staining to determine whether the chromosomal gaps and breaks increased in the ATR-downregulated cells during gene amplification at various MTX concentrations. Although more than 120 fragile sites have been identified in

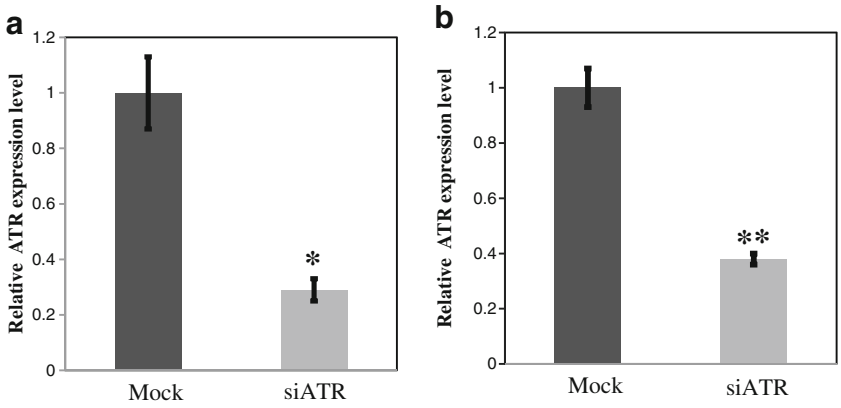

Fig. 2 Real-time RT-PCR analysis of ATR mRNA expression level in CHO-siATR and CHO-mock cells. Total RNA was isolated from each CHO cell pool (**a** after selection and **b** at 70 days culture after selection). CHO β-actin gene was used as an internal standard. The *error bars* represent the standard deviation (n=3). *P<0.05 and **P<0.01 vs. control, two-tailed unpaired t test

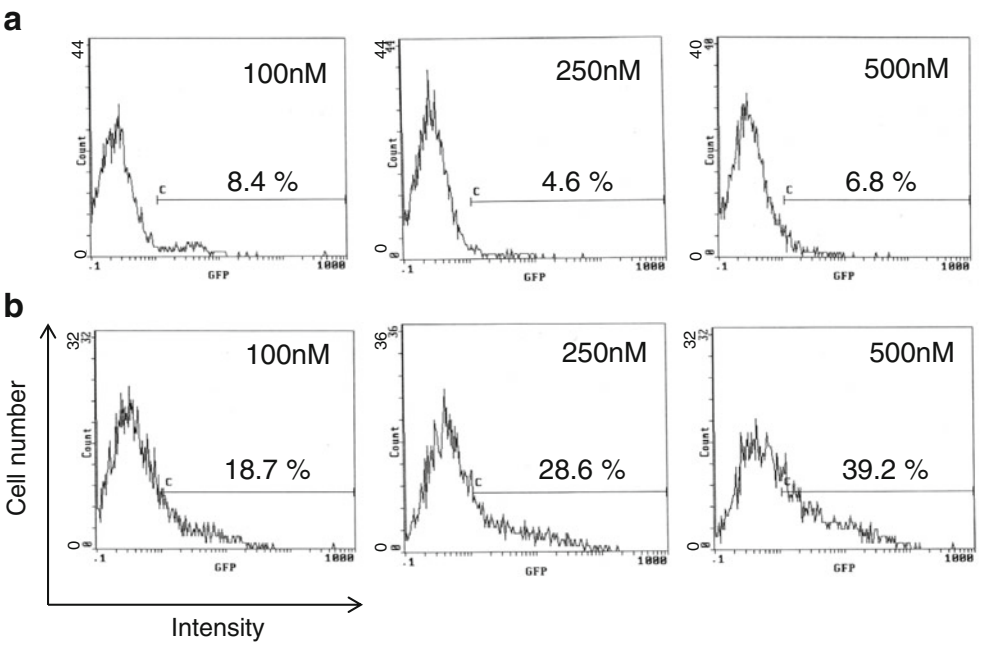

Fig. 3 Flow cytometry analysis of GFP-positive cells at end of first passage under 100, 250, and 500 nM MTX concentrations. The parental DG44 cells were used as a negative control to determine the GFP-positive gate (*C*). The GFP-positive gate (*C*) was set by excluding the area of nontransfected DG44 cells from the *x*-axis. On the basis of the gate (*C*), the ratio of GFP-positive cells was evaluated. **a** CHO-mock cell pool; **b** CHO-siATR cell pool

the human genome (Durkin and Glover 2007), there is not much fragile site information available in CHO cells. Recently, the genomic sequence of the CHO K1 cell was reported (Hammond et al. 2011; Xu et al. 2011). However, detecting fragile site expression using next-generation sequencing, bacterial or yeast artificial chromosome probe hybridization is inadequate. In this study, based on the fact that fragile sites are expressed as gaps, constrictions, or breaks on metaphase chromosomes (Lukusa and Fryns 2008), the number of gaps and breaks on metaphase chromosomes in CHO-siATR and CHO-mock cells was counted by direct counting under fluorescence microscopic observation. Each metaphase spread was prepared at the end of first cultivation of cells under 100-, 250-, and 500-nM MTX concentrations using a metaphase spreader (Cao et al. 2012b). As shown in Fig. 4, CHO-siATR cells showed significantly more chromosomal aberrations than did the CHO-mock cells during gene amplification by MTX treatment. While no gaps and breaks were observed in the mock cells at the 100- and 250-nM MTX concentrations, the CHO-siATR cells had a total of six and eight gaps or breaks, respectively ($n=50$ individual cells). Furthermore, there was a dramatic rise in the overall number of chromosomal gaps or breaks at the 500-nM MTX concentration; the CHO-siATR cells showed a total of 24 gaps or breaks, a much higher number of aberrations than the three gaps or breaks in the mock cells. These results were summarized as the average number of chromosomal aberrations per cell (Fig. 4). The increase of fragile site expression caused by ATR depletion was previously investigated in human cells (Casper et al. 2002). Our results confirmed the previous data for human cells. Moreover, the results showed that fragile site expression can be increased by ATR downregulation in CHO cells.

Comparison of IgG productivity

IgG-producing cell lines were generated to confirm the previous results obtained in GFP-producing cell lines. CHO-siATR and CHO-mock cells were super-transfected with both pcDNA 3.1-Neo-HC and pcDNA 3.1-Hyg-DHFR-LC encoding the heavy and light chains of an IgG, respectively. Pools of IgG-producing cells were generated under 50 nM MTX and 500 μg/mL G418 selection pressure and amplified at 100-nM MTX concentration. The amplified cells in both cell lines were then inoculated in six-well plates at 1×10^5 cells/well in 3-mL medium. The IgG-producing cells were evaluated in six-well plates using a replicate culture method. In brief, supernatants were taken every 24 h for 5 days for ELISA to determine antibody concentrations. The adherent cells in each well were detached by a trypsin treatment and viable cell concentrations were determined using an automatic cell counter, Vi-Cell (Beckman Coulter) every 24 h for 5 days. On the basis of time course of cell and antibody concentrations in six-well plate replicate culture, we evaluated their productivity. The ATR-downregulated cells showed a significant increase in specific production rate of an average of 0.08 pg cell^{-1} day^{-1}, which was approximately four times higher than the average of 0.02 pg cell^{-1} day^{-1} in the mock cells (Fig. 5). The volumetric productivity of each cell line was also investigated to evaluate the influence of ATR downregulation. As shown in Fig. 5, the volumetric productivity of ATR knockdown cells was an average of 0.035 mg L^{-1} day^{-1}, which was approximately three times higher than the average of 0.013 mg L^{-1} day^{-1} of the mock cells, suggesting that ATR knockdown generated the pool of higher-producing cells during the gene amplification process.

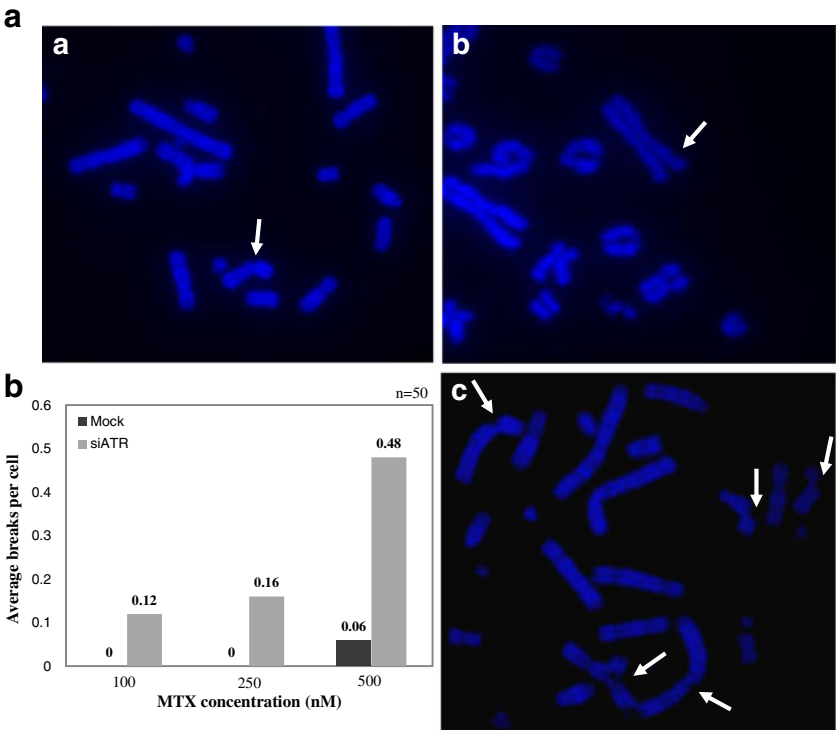

Fig. 4 **a** Representative aberrations of metaphase chromosomes in CHO-siATR cells during gene amplification process under MTX treatment at *a* 100-, *b* 250-, and *c* 500-nM concentrations. Metaphase chromosomes were prepared at the end of first cell culture under each MTX concentration. **b** Average chromosomal gaps and breaks per cell. $n=50$ metaphases for each cell line and condition were investigated

Estimation of amplified transgene copy number

Quantitative real-time PCR was used to estimate the amplified transgene copy number of GFP-producing cell lines during the gene amplification process. GFP-producing cells in both CHO-siATR and CHO-mock cell lines were cultured under MTX treatment until in a confluent state, and genomic DNAs were isolated before the first passage. The average copy number of ATR-downregulated cells was 15.4±0.8, 27.6±0.3, and 62.0±2.9 at 100-, 250-, and 500-nM MTX concentrations, respectively. These numbers were approximately 24 times higher than 3.98±0.09, 2.20±0.03, and 2.59±0.07 of the mock cells (Fig. 6). Interestingly, the amplified transgene copy numbers in the pools of ATR-downregulated cells were increased proportionally with the MTX concentration. The amplified transgene copy numbers in the IgG-producing cells were also investigated during the gene amplification process at 100-nM MTX concentration by the same protocol. The amplified light and heavy-chain copy numbers of the pool of ATR knockdown cells were 13.2±3.8 and 11.8±1.8, respectively, which were approximately seven times higher than 6.95±0.07 and 1.68±0.04 of the mock cells. The results from both the GFP- and IgG-producing cells showed that the pools of ATR-downregulated cells had much higher amplified transgene copy numbers as compared with the pools of mock cells during the gene amplification process.

Discussion

In the late 1980s, many researchers investigated the relationship between cell growth and antibody productivity in hybridoma

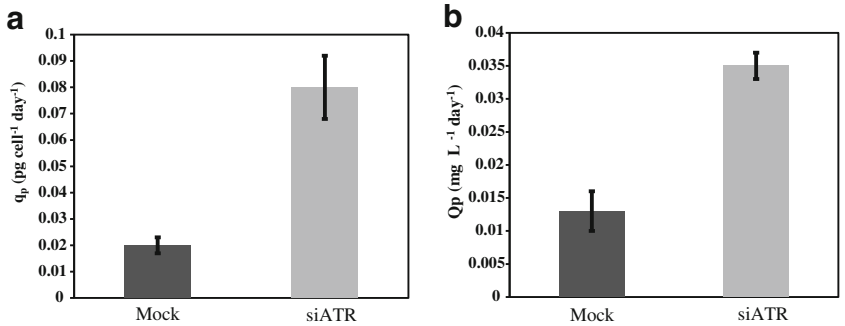

Fig. 5 **a** Specific production rate q_p (in picograms per cell per day) of CHO-mock and CHO-siATR cells producing IgG after gene amplification at 100-nM MTX concentration. **b** Average volumetric productivity Q_p (in milligrams per liter per day) of CHO-mock and CHO-siATR cells producing IgG after gene amplification at 100-nM MTX concentration. The *error bars* represent the standard deviation ($n=3$)

cells. Suzuki and Ollis (1989) pointed out the importance of cell cycle in antibody production. They focused on the trade-off relationship between specific growth and antibody production rates in hybridoma antibody production and constructed the cell cycle-based structure model to explain this phenomenon. Recently, Bi et al. (2004) focused on control of the cyclin-dependent kinase and overexpressed the cyclin-dependent kinase inhibitor, such as $p21^{CIP1}$ to achieve cell cycle arrest. The engineered CHO cells showed 4-fold higher productivity. Kuystermans and Al-Rubeai (2009) also tried to accelerate the cell cycle by overexpression of the *c-myc* gene in CHO cells. The overexpressed cells showed an increase of maximum cell density. Majors et al. (2008) were able to increase viable CHO cell density in serum-free suspension culture by overexpression of the EF2F-1 cell cycle transcription factor. Their cell cycle engineering approach can improve cell growth in commercially available cell cultures. However, no one has yet investigated cell cycle engineering focused on checkpoints for the construction of a highly productive CHO cell line. In this study, we investigated the relationship between the function of cell cycle checkpoint and gene amplification and showed the possibility of industrial applications of this novel method of accelerating gene amplification and quickly generating CHO cell lines having high productivity. We demonstrated that gene amplification can be rapidly induced by ATR downregulation.

The percentage of GFP-positive cells observed in the pool of ATR-downregulated cells was about two to six times higher than the percentage in the control group at the various MTX concentrations (Fig. 3). Analysis of transgene copy number revealed that the pool of ATR-downregulated cells had about a 4- to 24-fold higher transgene copy number as compared with that of the control group during gene amplification by MTX treatment (Fig. 4). Taken together with the concept that the level of gene expression is generally proportional to the copy number of a transgene (Guarna et al. 1995; Jiang et al. 2006; Pendse et al. 1992), these results suggest that ATR downregulation increased the generation of GFP-positive cells by accelerating gene amplification. However, we could not find out the reason why gene amplification process at higher MTX concentrations had failed to increase the transgene copy number and GFP-positive cells in the pools of mock cells. Many researchers reported the dominance of nonproducing mutant cells at high MTX concentrations (Chuck and Palsson 1992; Yoshikawa et al. 2001). Since we have used the pools of transfected cells and single-step amplification method in this research, it was estimated that nonproducing mutant cells became dominant in the mock pools. Further investigation is necessary to explain this phenomenon. DHFR-mediated gene amplification is a time-consuming and laborious process and one of the bottlenecks in establishing stable and high-producing cell lines. ATR downregulation weakens the cell cycle checkpoint functions and probably accelerates gene amplification via chromosome rearrangements. The rapid generation of cells with a higher transgene copy number could efficiently reduce the duration and labor needed for cell line establishment at early stages of gene amplification. Recently, many efficient gene expression technologies, e.g., use of chromatin opening elements, active promoter enhancer, and gene targeting transcriptionally active sites, have been used to develop high-producing cell lines without gene amplification process (O'Callaghan and James 2008). Nevertheless, DHFR-mediated gene amplification method still has high potential to increase productivity and is free from patent protection. Therefore, the improvement in gene amplification methods could be valuable for the industrial application.

ATR downregulation on mAb-producing cells also showed an increase of mAb productivity and transgene copy number. After gene amplification at 100-nM MTX concentration, specific production rate in the pool of ATR-downregulated cells was about four times higher than that of the mock cells, and the light- and heavy-chain copy numbers were about 1.9- and 6.5-fold higher, respectively, than were those of the mock cells (Figs. 5 and 6). These data are consistent with the results of GFP-producing cells. Moreover, volumetric productivity in the ATR-downregulated cell pool was also about three times higher than that of the mock cells, showing that ATR downregulation is also effective for enhancing volumetric productivity. Unfortunately, the specific production rate of constructed IgG-producing CHO cell was not high as compared with the value expected from the copy number of light and heavy chains. The reason for this phenomenon is not clear, but we assume that DNA sequence of our IgG may decrease the expression level of IgG. The expression level of recombinant proteins is affected by the nucleotide sequences of transfected genes. The sequences of heavy and light chains, which were used in this study, were directly obtained from phage-display libraries and not optimized for the expression of human IgG. The optimization of IgG sequence would be necessary for the improvement of the IgG expression.

Even though the precise mechanism of gene amplification remains elusive, Coquelle and his colleagues revealed that fragile site expression, which results in breaks at specific sites on metaphase chromosomes, plays an important role in triggering gene amplification by the BFB model (Coquelle et al. 1997). Cultured cells under MTX treatment are confronted with DNA replication stress during the *S*-phase of the cell cycle due to nucleotide depletion, which is caused by the inhibition of DHFR enzymes. Some specific regions of DNA may remain as a single-stranded state resulting from incomplete DNA replication. Fragile site expression is probably related to these single-stranded regions. Since cell cycle checkpoints strictly

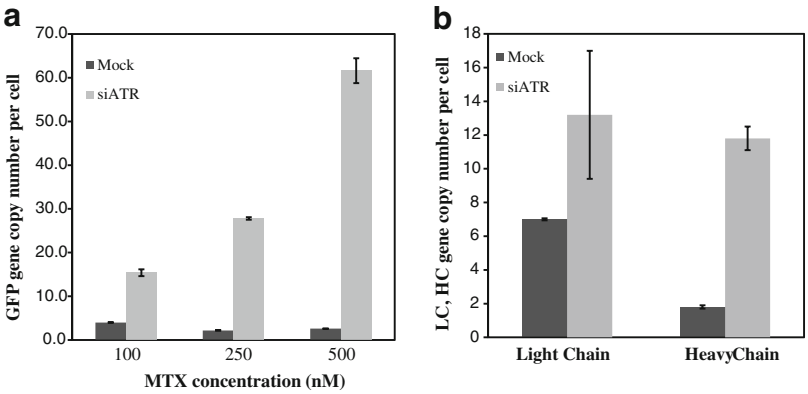

Fig. 6 Comparison of transgene copy numbers after gene amplification. **a** Absolute GFP gene copy numbers of CHO-mock and CHO-siATR cells. **b** Absolute HC and LC gene copy numbers of CHO-mock and CHO-siATR cells at 100-nM MTX concentration. Genomic DNA was isolated from each cell line after first culture under MTX treatment and analyzed by real-time quantitative PCR. The *error bars* represent the standard deviation (n=3)

control DNA damage to maintain chromosome integrity, most of the cells having damaged DNA may be arrested during the S- or G2-phase of the cell cycle or fail to proceed to metaphase and finally die under continuous nucleotide depletion. However, the cells that escape from the cell cycle checkpoint with single-stranded DNA and express chromatid breaks, may have a chance to amplify the exogenous *dhfr* gene to overcome replication stress. Casper and her colleagues revealed that one of the cell cycle checkpoint kinases, ATR, is critical for maintenance of fragile site stability, and that ATR deficiency results in fragile site expression (Casper et al. 2002). They also proposed a model of fragile site expression, in which fragile sites are unreplicated regions caused by stalled or collapsed replication forks that escape the ATR replication checkpoint.

ATR downregulation in CHO cells by the siRNA expression vector showed similar results of fragile site stability with Casper's previous study of the human HCT116 cell line (Casper et al. 2002). At various MTX concentrations, the ATR-downregulated cells expressed more breaks on metaphase chromosomes than did the control, and the frequency of breaks in ATR-downregulated cells increased at higher MTX concentrations. Although no breaks or only a few breaks were found in the control group after gene amplification at 100-, 250-, and 500-nM MTX concentrations, it is thought that fragile site expression may also have happened at early stages of MTX treatment in the control group. These results suggest that, while inducing gene amplification via expressed fragile sites may be limited in the mock cells, the ATR-downregulated cells may have enhanced gene amplification by increasing the fragile site expression.

Although we showed that ATR downregulation is an effective approach to accelerate gene amplification, the method used in this study would be better modified for industrial applications. We have used a constitutive expression of siRNA against ATR to evaluate the effect of ATR downregulation through gene amplification process which takes at least several weeks. In this study, we did not evaluate the genomic instability of ATR-downregulated CHO cells through a long-term culture. However, since genome integrity is strictly regulated by cell cycle checkpoint, ATR downregulation may affect the genomic stability of constructed CHO cells. Cell line stability is a critical factor for the commercial production. Recently, the inducible expression system, i.e., Tet-On/Off system, has been effectively used for cell line construction (Chung et al. 2004; Mohan et al. 2007). Thus, conditional expression systems (Gupta et al. 2004; Kappel et al. 2007) would be more rational to reduce ATR expression only during the phase of cell line development and recover the normal ATR expression level to maintain genome stability afterwards. Briefly, Tet-On system would be suitable to downregulate ATR expression level during cell line development stages. After the construction of stable cell lines using conditional expression plasmids, ATR expression level could be tightly downregulated in a dosage-dependent manner by the addition of tetracycline or doxycycline to accelerate gene amplification and then could be recovered by the removal of these antibiotics to prevent any drawbacks caused by genome instability.

In conclusion, we have demonstrated that gene amplification can be accelerated by the downregulation of a cell cycle checkpoint kinase, ATR, and a pool of high-producing cells can be rapidly derived in a short time after MTX treatment. This novel method focuses on generating much more high-producing cells in a heterogeneous pool as compared with the conventional method and would thus contribute to reducing the time and labor required for cell line establishment by increasing the possibility of selecting high-producing clones.

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