Repeated integration of antibody genes into a pre-selected chromosomal locus of CHO cells using an accumulative site-specific gene integration system

Yoshinori Kawabe · Hirokatsu Makitsubo ·
Yujiro Kameyama · Shuohao Huang ·
Akira Ito · Masamichi Kamihira

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Abstract We previously reported an accumulative site-specific gene integration system using Cre recombinase and mutated *loxP* sites, where a recombinase-mediated cassette exchange (RMCE) reaction is repeatable. This gene integration system was applied for antibody production using recombinant Chinese hamster ovary (CHO) cells. We introduced an exchange cassette flanked by wild-type and mutated *loxP* sites into the chromosome of CHO cells for the establishment of recipient founder cells. Then, the donor plasmids including an expression cassette for an antibody gene flanked by a compatible pair of *loxP* sites were prepared. The donor plasmid and a Cre expression vector were co-transfected into the founder CHO cells to give rise to RMCE in the CHO genome, resulting in site-specific integration of the antibody gene. The RMCE procedure was repeated to increase the copy numbers of the integrated gene. Southern blot and genomic PCR analyses for the established cells revealed that the transgenes were integrated into the target site. Antibody production determined by ELISA and western blotting was increased corresponding to the number of transgenes. These results indicate that the accumulative site-specific gene integration system

could provide a useful tool for increasing the productivity of recombinant proteins.

Keywords Accumulative site-specific gene integration · CHO cells · Cre-*loxP* · Recombinant antibody production · Recombinase-mediated cassette exchange

Introduction

Over the past two decades, cultured mammalian cells have become a typical platform for producing therapeutic proteins, because they can produce recombinant proteins with proper posttranslational modifications such as glycosylation and folding (Wurm 2004). However, the production cost of recombinant proteins using cultured mammalian cells is very high, therefore there is a requirement to increase the yield of target proteins but reduce the associated costs. Optimization of culture conditions, development of effective culture medium and expression systems, genetic modification of host cells, and amplification of target genes in the host genome have been investigated in attempts to enhance productivity (Dyck et al. 2003). The amplification of target genes in the host genome using selectable marker genes involved in antibiotic resistance and nucleotide metabolism are frequently applied for the generation of producer cells. As an example, following transfection

Y. Kawabe · H. Makitsubo · Y. Kameyama ·
S. Huang · A. Ito · M. Kamihira (✉)
Department of Chemical Engineering,
Faculty of Engineering, Kyushu University,
744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan
e-mail: kamihira@chem-eng.kyushu-u.ac.jp

with a plasmid containing expression cassettes for dihydrofolate reductase (*DHFR*) and other target genes into *DHFR*-deficient Chinese hamster ovary (CHO) cells, such as CHO-DG44 cells (Urlaub and Chasin 1980), the copy number of the target gene is amplified in the genome by gradually increasing the concentration of methotrexate (MTX) in the culture medium (Jun et al. 2005). The efficiency of recombination and amplification of target genes is generally low and highly dependent on the type of cell used. Thus, the establishment of high-producer cells is a time-consuming and labor-intensive process that requires the screening of many cells. Furthermore, since the integrated position of the target gene into the chromosome cannot be controlled, the level of transgene expression can be highly dependent on the site of integration. In some cases, transgene expression is diminished or disappears altogether during the culture period (Wilson et al. 1990; Kwaks and Otte 2006). Targeted integration of a transgene into a predetermined chromosomal site is desired for stable expression of recombinant proteins.

Of the site-specific gene recombination systems, the Cre-*loxP* system has been well studied and frequently used for animal cells (Nagy 2000). Cre recombinase derived from bacteriophage P1 catalyzes a recombination reaction between two *loxP* target sites. A *loxP* site is defined as a 34 bp DNA sequence composed of an 8 bp spacer region flanked by two identical 13 bp inverted repeats (arm regions). The excision, integration, inversion and exchange reactions that occur depend on the number and direction of inserted *loxP* sites. During the recombination processes, the excision reaction is kinetically favored. In order to alter the reaction kinetics, many mutated *loxPs* have been developed. Mutations in the spacer and arm regions result in two types of mutated *loxPs*, spacer and arm mutants, respectively. The spacer mutants, represented by *lox511* and *lox2272*, are characterized by controlling the specificity of the reaction. These types of mutated *loxPs* have been used for recombinase-mediated cassette exchange (RMCE), in which a gene flanked by two non-recombining target sites is replaced with another gene flanked by the compatible target sites (Kolb 2001). Arm mutants, represented by *lox66* and *lox71*, have been employed to promote integration reactions, because a non-reactive *loxP* site is generated following a recombination reaction between arm-mutated *loxPs* with mutation in an opposite arm (Oberdoerffer et al. 2003).

In a previous study, we reported an accumulative site-specific gene integration system by RMCE using Cre recombinase and mutated *loxPs* (Kameyama et al. 2010). This system enables repeated integration of multiple genes by combining the characteristics of mutated *loxPs*. In the present study, we applied this gene integration system for the generation of recombinant CHO cells producing a single-chain antibody fragment fused with the Fc-region of immunoglobulin G (scFv-Fc), as well as a whole antibody composed of heavy and light chains (chimeric immunoglobulin G, IgG). The integration site of the transgene was confirmed by genomic polymerase chain reaction (PCR) and Southern blotting, and recombinant protein production was evaluated by enzyme-linked immunosorbent assay (ELISA) and western blotting. The accumulative gene integration system provides a site-specific gene amplification procedure for generating cells able to produce recombinant proteins.

Materials and methods

Cells and culture media

The CHO-K1 cells (RIKEN, Tsukuba, Japan) were cultured in an adherent condition using Ham's F12 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 units mL⁻¹ penicillin G potassium and 100 µg mL⁻¹ streptomycin sulfate (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C in a 5% CO₂ incubator. For preparing western blot samples, CHO cells were cultured in serum-free medium (CHO-S-SFM; Invitrogen, Carlsbad, CA, USA) to eliminate the effect of serum proteins.

Plasmid construction

DNA sequences of the *loxP* sites used in this study were described in our previous report (Kameyama et al. 2010). A red fluorescent protein (DsRed) gene fragment derived from pIRES2-DsRed-Express (Clontech, Palo Alto, CA, USA) was ligated into *NotI*-digested pcDNA4/*loxP-loxP1* (P1) (Kameyama et al. 2010) to generate pcDNA4/*loxP*-DsRed-*loxP1* (P1/DsRed). This plasmid contains a zeocin resistant gene as a selection marker.

A blasticidin resistant gene (*Bla^r*) fragment was amplified from pLenti6/V5/GW/lacZ (Invitrogen) using the primers 5'-GCG GTA CCA TGG CCA AGC CTT T-3' and 5'-GAA GAT CTT TAG CCC TCC CAC ACA TAA-3' in order to append *KpnI* and *Bgl/II* digestion sites (underlined), respectively, onto either end of the PCR product. The PCR was initiated using KOD plus DNA polymerase (Toyobo, Osaka, Japan) and incubating at 94 °C for 2 min, followed by 30 cycles of amplification at 94 °C for 15 s, 60 °C for 30 s and 68 °C for 30 s. The PCR product was digested with the relevant restriction enzymes and ligated into *KpnI* and *BamHI*-digested pCEP4 (Invitrogen) to generate pCEP4/*Bla^r*. The DNA sequences were confirmed using a DNA sequencer (Prism 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The pBlue/*loxP-loxP4-IRES/EGFP-loxP2* (P2) construct (Kameyama et al. 2010), which included an internal ribosomal entry site (*IRES*) sequence and an enhanced green fluorescent protein (*EGFP*) gene flanked by *loxP* sites was digested with *NotI*. The DNA fragment was treated with Klenow enzyme (Nippon Gene, Toyama, Japan) to make blunt-end fragments. The DNA fragment encoding the *Bla^r* gene and SV40 polyA signal region prepared from pCEP4/*Bla^r* was ligated into the blunt-ended P2 after digesting with *NotI* to generate pBlue/*loxP-Bla^r/polyA-loxP4-IRES/EGFP-loxP2*. The DNA fragment encoding the CMV promoter and SV40 polyA signal region prepared from pCEP4 was ligated into the blunt-ended pBlue/*loxP-Bla^r/polyA-loxP4-IRES/EGFP-loxP2* after digesting with *BamHI* and *HindIII* to generate pBlue/*loxP-Bla^r/polyA-loxP4-CMV/polyA-loxP2*. An anti-prion single chain Fv fused with the Fc region derived from the human IgG1 (scFv-Fc) gene fragment in pMSCV/GΔAscFv-Fc (Kamihira et al. 2005) was ligated into the *HindIII*-digested pBlue/*loxP-Bla^r/polyA-loxP4-CMV/polyA-loxP2* to generate pBlue/*loxP-Bla^r/polyA-loxP4-CMV/scFv-Fc/polyA-loxP2* (P2/scFv-Fc). The anti-human CD2 of the light (L)-chain gene fragment derived from pMSCV/GΔALIH (Kamihira et al. 2009) was ligated into the blunt-ended pBlue/*loxP-Bla^r/polyA-loxP4-CMV/polyA-loxP2* after digesting with *SalI* to generate pBlue/*loxP-Bla^r/polyA-loxP4-CMV/L/polyA-loxP2* (P2/L).

A neomycin resistant gene (*Neo^r*) fragment was prepared from pBlue/*loxP-Neo^r-loxP1-IRES/DsRed-loxP5* (P3Neo^r) (Kameyama et al. 2010) by digesting

with *BamHI* and was ligated into the *BamHI*-digested pCEP4 to generate pCEP4/*Neo^r*. The P3Neo^r construct was digested with *XbaI* and self-ligated to generate P3Δ in which the *IRES* region and *DsRed* gene are deleted. The fragments encoding the *Neo^r* and SV40 polyA signal regions prepared from pCEP4/*Neo^r* were ligated into the blunt-ended P3Δ after digesting with *NotI* to generate pBlue/*loxP-Neo^r/polyA-loxP1-loxP5*. A DNA fragment encoding the CMV promoter and SV40 polyA signal region prepared from pCEP4 was ligated into the blunt-ended pBlue/*loxP-Neo^r/polyA-loxP1-loxP5* after digesting with *HindIII* to generate pBlue/*loxP-Neo^r/polyA-loxP1-CMV/polyA-loxP5*. The anti-prion scFv-Fc and anti-human CD2 of the heavy (H)-chain gene fragments derived from pMSCV/GΔAscFv-Fc and pMSCV/GΔALIH, respectively, were ligated into the *HindIII*-digested pBlue/*loxP-Neo^r/polyA-loxP1-CMV/polyA-loxP5*. The resultant plasmids were designated pBlue/*loxP-Neo^r/polyA-loxP1-CMV/scFv-Fc/polyA-loxP5* (P3/scFv-Fc) and pBlue/*loxP-Neo^r/polyA-loxP1-CMV/H/polyA-loxP5* (P3/H), respectively.

Schematic drawings of the plasmid constructs used as gene donors are shown in Fig. 1.

Generation of recombinant CHO cells producing antibodies

Integration of genes encoding various antibodies into the genome of CHO cells using the Cre-mediated accumulative gene integration system was performed as described previously (Kameyama et al. 2010). Recipient founder cell lines containing the P1/*DsRed* plasmid sequence in the genome were established. The linearized P1/*DsRed* plasmid was transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, the cells were cultured in a selective medium containing 200 μg mL⁻¹ zeocin (Invitrogen) to screen for stable transformants. For the red fluorescent clones, genomic integration of the plasmid sequence was confirmed by PCR and Southern blot analyses. A CHO cell line (CHO/P1[*DsRed*]) with a single copy of the transgene integrated into the genome was selected and used as founder cells for accumulative gene integration.

The scheme for accumulative gene integration of antibody genes into CHO/P1[*DsRed*] cells by RMCE has been summarized in Fig. 1. For transfection of donor plasmids in each RMCE reaction, the cells were seeded at a density of 1.4 × 10⁵ cells

Fig. 1 Schematic drawing of the integration procedure for antibody genes by the Cre-mediated accumulative gene integration system. *loxP* target sites are represented by rectangular boxes. Wild-type and the two mutated spacers of *loxP* target sites are indicated by a triangle, circle and square in the center box, respectively. The mutated arms are represented by the diagonally-striped box. The *loxPs* with double-mutated arms generated after recombination are no longer associated

in 0.5 mL medium in 24-well tissue culture plates. At 24 h after cell seeding, the cells were co-transfected with a Cre expression vector (pxCAN-Cre, 20 ng) (Kanegae et al. 1995) and the donor plasmid (800 ng) using a lipofection reagent. For the first RMCE reaction in the cycle of gene integration, P2/scFv-Fc or P2/L was used as the donor plasmid. For the second RMCE reaction, P3/scFv-Fc or P3/H was used as the donor plasmid. After 10–14 days of culture in selection medium containing 5 $\mu\text{g mL}^{-1}$ blasticidin S (Invitrogen) for P2/scFv-Fc and P2/L, or 500 $\mu\text{g mL}^{-1}$ G418 (Sigma-Aldrich) for P3/scFv-

with Cre-mediated recombination. *Bla^r*, blasticidin resistance gene; *Neo^r*, neomycin resistance gene; P_{CMV} , human cytomegalovirus (CMV) immediate-early promoter/enhancer; *scFv-Fc*, chicken anti-prion single-chain-antibody-fragment gene fused with the Fc region gene from human IgG1; *L-chain*, anti-human CD2 chimeric antibody light chain gene; *H-chain*, anti-human CD2 chimeric antibody heavy chain gene; *pA*, SV40 polyadenylation signal sequence; *Zeo^r*, zeocin resistance gene

Fc and P3/H, colonies were picked for establishing cell clones. For the third RMCE reaction, P2/scFv-Fc was used as the donor plasmid, and cells were selected with the same procedure as the first RMCE reaction.

Genomic PCR and Southern blot analyses

Genomic DNA extracted from cells after each RMCE reaction step, using a genomic DNA preparation kit (Magextractor; Toyobo), was subjected to genomic PCR and Southern blot analysis.

The specific sequences to detect site-specific recombination were amplified by PCR from genomic DNA as the template. The primers used are summarized in Table 1. The PCR was initiated with DNA polymerase (G-Taq; Cosmo Genetech, Seoul, Korea) at 95 °C for 2 min, followed by 35 cycles of amplification at 95 °C for 30 s, 55–57 °C for 40 s, 72 °C for 15–70 s and 72 °C for 5 min for final extension.

For Southern blot analysis, genomic DNA (10 µg) digested with suitable restriction enzymes was electrophoresed on a 0.8% (w/v) agarose gel and then transferred to a nylon membrane (Hybond-XL; GE Healthcare, Buckinghamshire, UK). The membrane was incubated with DsRed and scFv-Fc probes prepared from P1/DsRed and pMSCV/GΔAscFv-Fc, respectively. The preparation of probes, hybridization procedure and detection of hybridized signals were performed using a commercially available kit (DIG-High Prime DNA Labeling and Detection Starter Kit II; Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Determination of cell growth and recombinant antibody production rates

Recombinant CHO cells (2.5×10^4 cells/well) generated from each RMCE reaction were seeded in 0.5 mL of the serum-containing medium in 24-well plates (Greiner bio-one, Frickenhausen, Germany) and cultured in adherent condition for 6 days. Half volume of the medium was replaced with equal volume of fresh medium every day. The wells for cell growth measurement were prepared in triplicate for each condition. Viable cell density was determined by the

trypan blue exclusion method. A solid-phase enzyme-linked immunosorbent assay (sandwich ELISA) was performed to measure the antibody concentration in culture medium as described previously (Kamihira et al. 2005, 2009). The rabbit IgG fraction of anti-human IgG (Fc) (Rockland Immunochemicals, Philadelphia, PA, USA) and rabbit peroxidase (POD)-conjugated anti-human IgG antibodies (Rockland Immunochemicals) were used as primary and secondary antibodies, respectively. Calibration curves were created using a dilution series of human Fc fragment (Jackson ImmunoResearch, West Grove, PA, USA) or purified scFv-Fc (Kamihira et al. 2005). The antibody concentration was expressed as mean values with standard deviations.

Western blot analysis

Reducing and non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis was performed to detect the recombinant antibodies, as described previously (Kamihira et al. 2009). The human Fc fragment (Jackson ImmunoResearch) and human IgG (Sigma-Aldrich) were used as positive controls. For the detection of chimeric antibody, a small but sufficient amount of staphylococcal protein A-Sepharose beads (rProtein A-Sepharose™ Fast Flow; GE Healthcare, Fairfield, CT, USA) was added to the samples cultured using serum-free media. The mixture was incubated at 4 °C for 3–4 h with gentle mixing. The Sepharose beads were collected by centrifugation ($100 \times g$, 5 min, 4 °C) and washed five times with phosphate-buffered saline (PBS). After the beads were mixed with SDS-PAGE sample buffer with or without 2-mercaptoethanol and boiled at 100 °C for 5 min, the supernatant was subjected to SDS-PAGE on a 7.5% (w/v) polyacrylamide gel. For the detection of scFv-Fc, the samples were boiled in SDS-PAGE sample buffer with 2-mercaptoethanol and subjected to SDS-PAGE on a 10% (w/v) polyacrylamide gel. After transferring onto polyvinylidene fluoride membranes (GE Healthcare), the membranes were soaked in Tris-buffered saline-Tween 20 containing 5% (w/v) non-fat milk at 4 °C overnight. Recombinant antibody proteins on the membranes were detected using a rabbit POD-conjugated anti-human IgG antibody (Rockland Immunochemicals). The specific antibody-antigen

Table 1 Primers for genomic PCR analysis

Primer label	Sequence (5' → 3')
F	TAG AAG ACA CCG GGA CCG AT
R	ACA GTG GGA GTG GCA CCT T
B	GAA GAT CTT TAG CCC TCC CAC ACA TAA
A	GTG GAG GTG CAT AAT GCC AA
L	TTA CGA GAA ACA CAA AGT CTA CGC
N	GCA TCA GAG CAG CCG ATT GT
H	GTG GAG GTG CAT AAT GCC AA
C	GCG GAA CTC CAT ATA TGG GC

complexes were detected using an enhanced chemiluminescence kit (GE Healthcare).

Results

Establishment of recipient cells and recombinant antibody producer cells

Recombinant CHO cells (CHO/P1), in which *loxP* target sites were introduced into the genome, were established (Kameyama et al. 2010). To facilitate the evaluation of the first RMCE reaction, we constructed a recipient plasmid (P1/DsRed) containing an expression cassette with the DsRed gene flanked by wild-type *loxP* and mutated *loxP* (*loxP1*), and the linearized plasmid was then transfected into CHO-K1 cells. At 48 h post-transfection, the cells were cultured in a selective medium for 10–14 days. Twenty-four clones were picked, based on DsRed expression (Fig. 2a). Genomic DNA extracted from the clones was subjected to PCR (Fig. 2c) to detect genomic integration of the P1/DsRed sequence. A cell clone harboring a single copy of the *loxP* sites was chosen as the recipient founder cells (CHO/P1[DsRed]) for accumulative gene integration of antibody genes. The single-copy integration of transgene in the genome was confirmed by Southern blot analysis (Fig. 2d).

In order to generate producer cells of recombinant antibodies using the accumulative gene integration system, we constructed donor plasmids incorporating an expression unit of recombinant antibody genes flanked by *loxPs* (Fig. 1). A scFv-Fc gene was used for repeated integration of the target gene, and a chimeric antibody gene composed of L- and H-chain genes was used for whole antibody (chimeric IgG) production by RMCE-mediated integration of the L-chain gene followed by the H-chain gene. For the introduction of the scFv-Fc gene, the integration reaction was repeated three times.

For the first RMCE reaction, CHO/P1[DsRed] cells were co-transfected with P2/scFv-Fc or P2/L plasmid and a Cre expression vector and then cultured in the presence of blasticidin for 10–14 days. The expected gene structures after P2/scFv-Fc and P2/L integration by RMCE are shown in Figs. 3a and 4a, respectively. Around 50% of cells lost their red fluorescence, suggesting successful genomic integration. Cell clones were isolated by the limiting dilution method. Among

the established clones, 87.5% (7/8) scFv-Fc and 100% (4/4) L-chain clones were confirmed as the expected clones by genomic PCR using specific primer sets (Figs. 3d, 4c). These clones were designated CHO/scFv-Fc x1 and CHO/L for the scFv-Fc and L-chain of anti-CD2, respectively, and used in the next RMCE reaction. Clones without the Cre expression plasmid did not result in amplification of specific DNA fragments.

For the second RMCE reaction, CHO/scFv-Fc x1 or CHO/L cells were co-transfected with P3/scFv-Fc or P3/H plasmid, respectively, and the Cre expression plasmid. The cells were cultured in the presence of G418 for 10–14 days. The expected gene structures after P3/scFv-Fc and P3/H integration by RMCE are shown in Figs. 3b and 4b, respectively. Cell clones obtained by the limiting dilution method were subjected to genomic PCR using the specific primer pairs F and N, and H and C to check the site-specific integration (Figs. 3e, 4d). For the complete RMCE clones, DNA fragments were not amplified using primers F and B. In all established clones (7 out of 7 clones for scFv-Fc and 6 out of 6 clones for H-chain), DNA fragments were amplified using primers F and N but not amplified using primers F and B (data not shown), indicating that the second RMCE reaction completed at the target *loxP* sites. The clones established after the second RMCE were designated as CHO/scFv-Fc x2 and CHO/HL for scFv-Fc and anti-CD2, respectively, and used for further experiments.

In this accumulative gene integration system, a set of reactive *loxP* sites (*loxP*[wt] and *loxP1*) in the genome reverted to the original set after the second RMCE reaction, therefore gene integration would be repeatable. We performed the third RMCE reaction for scFv-Fc integration using the P2/scFv-Fc plasmid. After the RMCE reaction, the expected transgene structure in the genome is shown in Fig. 3c. In the presence of the Cre expression vector, a DNA fragment of expected size was amplified using primers F and B, whereas no amplification was observed when the Cre expression vector was absent (Fig. 3f). After the RMCE reaction mediated by Cre, six clones established by the limiting dilution method in the presence of blasticidin were subjected to genomic PCR. All clones exhibited an amplicon when primers F and B were utilized, but no band was observed when using primers F and N (Fig. 3f), indicating that the third RMCE reaction also successfully occurred at the target *loxP* sites. These clones (CHO/scFv-Fc x3) were used for further experiments.

Fig. 2 Establishment of recipient founder cells.

a Phase contrast (*left*) and fluorescent (*right*) microscopic images of CHO/P1[DsRed] cells. **b** Schematic drawing of transgene structure in the genome of CHO/P1[DsRed]. Locations of primers (F and R) for genomic PCR are indicated as *arrows*. **c** Genomic PCR analysis. Genomic integration of the P1/DsRed sequence was confirmed by genomic PCR using primers F and R. Lane M, *Hinc*II-digested ϕ X174 and *Hind*III-digested λ DNA molecular weight marker; 1 H₂O, 2 parental CHO-K1, 3 CHO/P1[DsRed]. **d** Southern blot analysis. Genomic DNA digested with *Bgl*II was subjected to Southern blotting using a DsRed probe

To confirm that the integrated transgenes were site-specifically introduced into the genome, Southern blot analysis was performed on the established clones (Fig. 3g for CHO/scFv-Fc x1, x2 and x3). As shown in Fig. 3g, single bands with the expected size were detected for CHO/scFv-Fc x1 and 2. For CHO/scFv-Fc x3, although single band with the expected size was detected as a major band, minor bands were also detected. Since the minor bands were not dense, we think that they are non-specific artifacts. To further confirm the site-specific integration of the target gene for the established clones, we performed genomic PCR using some primer sets to detect random integration, but no bands were amplified for the clones (data not shown), indicating that the site-specific integration occurred in the clones established by RMCE reaction.

Production of recombinant antibody

The established scFv-Fc producer cells (CHO/scFv-Fc x1, x2 and x3) and founder cells (CHO/P1[DsRed]) were cultured for 6 days, and the cell growth and

productivity of scFv-Fc were evaluated. No significant difference in cell growth was observed among the cells (Fig. 5a). The scFv-Fc concentration in culture broth of the producer cells was determined by ELISA and the expression level of scFv-Fc increased during culture period. The cumulative scFv-Fc amounts throughout the culture period were 16, 22 and 29 μ g for CHO/scFv-Fc x1, x2 and x3, respectively, and the cell specific productivities for each cell line were 20, 29 and 40 $\text{pg cell}^{-1} \text{ day}^{-1}$, respectively. Thus, the production rate of scFv-Fc was increased with the copy number of the transgene (Fig. 5b). The expression level and molecular weight of the product were confirmed by western blot analysis (Fig. 5c). The scFv-Fc produced by the cells was detected at the expected size as described in the previous report (Ono et al. 2003) and the band intensity was also enhanced corresponding to the copy number of the transgene.

Western blot analysis was also performed to evaluate the expression of the chimeric antibody. As expected, CHO/L cells containing an integrated expression unit corresponding to the L-chain,

Fig. 3 Generation of scFv-Fc producer cells using an accumulative gene integration system. **a–c** Schematic drawing of transgene structures in the genome of scFv-Fc producer cells (**a** CHO/scFv-Fc x1, **b** CHO/scFv-Fc x2, **c** CHO/scFv-Fc x3). Location of primers (F and B, and A and R for CHO/scFv-Fc x1; F and N for CHO/scFv-Fc x2; F and B for CHO/scFv-Fc x3) are indicated by arrows. **d–f** Genomic PCR analysis after the first (**d**), second (**e**) and third (**f**) RMCE reactions. After each RMCE reaction, clones were established in selective medium containing the relevant antibiotics for screening. Lane M, *HincII*-

digested ϕ X174 and *HindIII*-digested λ DNA molecular weight markers; 1 H₂O, 2 CHO/P1[DsRed] (**d**), CHO/scFv-Fc x1 (**e**) or CHO/scFv-Fc x2 (**f**), 3 without Cre vector, 4 established clones, CHO/scFv-Fc x1 (**d**), CHO/scFv-Fc x2 (**e**) or CHO/scFv-Fc x3 (**f**). **g** Southern blot analysis. Genomic DNA samples digested with *EcoRI* and *ApaI* were subjected to Southern blotting using a scFv-Fc probe. Lane 1 CHO/P1[DsRed], 2 CHO/scFv-Fc x1, 3 CHO/scFv-Fc x2, 4 CHO/scFv-Fc x3

produced L-chain protein. The CHO/HL cells with integrated expression units of H- and L-chains produced both the H- and L-chain proteins (Fig. 5d). Under non-reducing conditions, a single band with molecular weight of 180 kDa was detected in the CHO/HL culture supernatants (Fig. 5e). The chimeric antibody productivity of CHO/HL cells was 0.065 pg cell⁻¹ day⁻¹ (data not shown).

Discussion

CHO cells are frequently used as host cells for the production of therapeutic proteins, as the cells can synthesize exogenous proteins with posttranslational modifications such as correct folding and glycosylation, features that are necessary to confer their biological activities. To establish highly productive

Fig. 4 Generation of anti-CD2 chimeric antibody producer cells by an accumulative gene integration system.

a, b Schematic drawing of transgene structures in the genome of L-chain producer cells (**a** CHO/L) and chimeric antibody producer cells (**b** CHO/HL). **c, d** Genomic PCR analysis after first (**c**) and second (**d**) RMCE reactions. Lane *M*, *Hinc*II-digested ϕ X174 and *Hind*III-digested λ DNA molecular weight markers; 1 *H*₂O, 2 CHO/P1[DsRed] (**c**) or CHO/L (**d**), 3 without Cre vector, 4 established clones, CHO/L (**c**) or CHO/HL (**d**)

Fig. 5 Cell growth and production of recombinant antibodies. **a** Growth curves of scFv-Fc producer cells. **b** Specific scFv-Fc production rate. **c–e** Western blot analysis of scFv-Fc (**c**) and chimeric antibody (**d, e**). **c** Cultures of CHO/P1[DsRed] (lane 1), CHO/scFv-Fc x1 (lane 2), CHO/scFv-Fc x2 (lane 3) and CHO/scFv-Fc x3 (lane 4) were subjected to reducing SDS-PAGE. **d, e** Cultures of CHO/P1[DsRed] (lane 1), CHO/L (lane 2) and CHO/HL (lane 3) were subjected to reducing (**d**) or non-reducing (**e**) SDS-PAGE

cells, gene amplification using a drug resistant gene has been applied to increase the copy number of transgenes leading to enhanced productivity of target proteins (Hacker et al. 2009).

In the present study, the Cre-mediated accumulative gene integration system we previously developed (Kameyama et al. 2010) was applied to recombinant antibody production. Successive gene integration of antibody genes into the target site was successfully achieved. When three copies of the gene expression unit of scFv-Fc were integrated into the genome, the production of scFv-Fc corresponded to an increase in the copy number of integrated transgenes. Although it has been reported that adjacent identical promoters often affect gene expression, known as transcriptional interference (Hasegawa and Nakatsuji 2002), significant gene suppression of target proteins was not observed even if the same expression unit (scFv-Fc) was tandemly introduced. It has previously been reported that exogenous promoter-based expression levels can be influenced by transcriptional orientation (Shearwin et al. 2005; Nyabi et al. 2009). Optimal orientation for multiple expression units should be considered to achieve higher expression of the transgene.

In this study, the antibody production of cells was measured within three passages from cell establishment by cloning after RMCE reaction and the cells were cultured without antibiotic pressure. Although we did not examine genomic stability of transgenes for long-term culture, suppression of transgene expression was not observed after long-term preservation (over a year). It was reported that the tandem integration of transgene induces instability of transgene in the genome (Garrrick et al. 1998) and an extensive chromosome rearrangement possibly occurs in CHO cells (Yoshikawa et al. 2000). To prevent transgene instability, the protective *cis*-regulatory elements (Kwaks and Otte 2006) may be applicable to genomic integration of multiple transgene cassettes by repeated RMCE reaction.

Producer cells established by the conventional gene amplification procedure possess a high transgene copy number of greater than 100 (Weidle et al. 1988). However, the chromosomal location of the transgene demonstrates variety and all exogenous gene expression units integrated into the genome do not exhibit similar expression levels. Campbell et al. (2010) reported that cells generated by random integration

exhibited a wide range of expression levels among clones, while the expression level was relatively uniform and stable among clones when a transgene was integrated into a pre-selected chromosomal locus. Thus, chromosomal location of the transgene influences the transgene expression level and stability of expression (Kwaks and Otte 2006). Although the chromosomal position of the transgene was not determined, the founder cells (CHO/P1[DsRed]) used in this study showed relatively high and stable expression of the DsRed transgene and of the introduced antibody genes mediated by Cre. The founder cells were screened in terms of high and stable expression of DsRed and single copy of the transgene, including the target *loxP* sites. This might be beneficial for this site-specific gene integration system, since the introduction of the next gene can be targeted to the chromosomal position exhibiting high and stable transgene expression. This gene integration system can be applied for targeting of known chromosomal recombination hotspots, such as *ROSA26* (Zambrowicz et al. 1997; Irion et al. 2007). In such cases, the *loxP* target sites have to be introduced to the hotspot by homologous recombination. If the *loxP* target sites are introduced into a vector for chromosomal hotspot screening, the gene integration system can be applied immediately after screening, enabling integration of a target gene into the hotspot. Besides hotspot targeting, the utilization of insulator sequences such as the locus control region (Ellis et al. 1996) and matrix attachment region (McKnight et al. 1996) may be effective for the stable expression of the transgene leading to a high production yield of target proteins after accumulative gene integration.

For applying the conventional gene amplification procedure using *DHFR* and MTX, it takes around 6 months to build up producer cells (Crouse et al. 1983). In this study, the donor plasmids containing a target gene expression unit and an antibiotic resistant gene flanked by *loxPs* were used to generate producer cells (Fig. 1). The time frame for a round of cell generation process including transfection for RMCE, drug selection and cloning was 20–30 days. We confirmed that Cre-mediated integration was completed within 48 h post-transfection (data not shown). The cell screening (drug selection and cloning) was the most time-consuming process in this study. If fluorescent genes are used as selection markers instead of drug resistant genes, the process time for cell

establishment may be greatly shortened by using a FACS device. In addition to improvement of the screening procedure, the selection of a mutated *loxP* target site and the number of target sites integrated into the founder cell genome may also contribute to improve the efficiency for generating high producer cells.

As a model for application of the accumulative gene integration system to multimeric protein production, genes for an anti-CD2 chimeric IgG were integrated into cellular chromosomes. Since IgG is composed of two polypeptides (H- and L-chains), the corresponding two genes have to be expressed in recombinant cells at the same time to produce intact antibodies. Therefore, expression units for L- and H-chains were consecutively integrated into cell genome so that the antibody molecule assembled as a hetero-tetramer after two RMCE reactions. In the practical application of antibody production, expression of H- and L-chain genes using one-packed vectors are driven by independent promoters or mediated by the IRES (Hotta et al. 2004; Kamihira et al. 2009) or 2A sequence (Fang et al. 2005). Although the productivity of chimeric antibody was similar to that of the previous report using producer cells with lower gene amplification (O'Callaghan et al. 2010), it was considerably low compared with that of scFv-Fc despite that the same chromosomal locus was used for integration. Since we have not optimized the expression units for H- and L-chain genes of the antibody, the expressions of H- and L-chain genes might be unbalanced. Thus, optimization of the expression units should be examined to improve the antibody productivity.

The product yield of high-producer CHO cells generated by the conventional gene amplification procedure reached up to 7.5 g L^{-1} of antibody concentration in the medium and the cell specific productivity was maintained at $55 \text{ pg cell}^{-1} \text{ day}^{-1}$ in the production phase (Yu et al. 2011). The scFv-Fc productivity in this study ($40 \text{ pg cell}^{-1} \text{ day}^{-1}$ for CHO/scFv-Fc x3) was comparable to that of cells generated by the conventional gene amplification procedure.

Using a recombinase-mediated integration system, some research groups reported recombinant protein production by CHO cells where a gene of interest was integrated into a pre-selected chromosomal locus (Kito et al. 2002; Wiberg et al. 2006; Huang et al.

2007; Zhou et al. 2010). Nehlsen et al. (2009) also reported antibody gene integration into CHO cells using Flp-RMCE. Recently, site-specific integration into a target locus was applied for the evaluation of packaging cell lines producing retroviral vectors with high and predictable titer (Gama-Norton et al. 2010) and for the establishment of recombinant G-protein-coupled receptor-expressing cell lines (Schucht et al. 2011). In these studies, however, a transgene cassette was introduced into the genome only once.

In conclusion, we applied an accumulative gene integration system to antibody production using recombinant CHO cells. The antibody genes were successively integrated into a predetermined site of the CHO genome by Cre recombinase-mediated cassette exchange. The production of recombinant antibody increased as the number of integrated genes increased. These results indicate that an accumulative site-specific gene integration system is applicable for gene amplification and production of recombinant proteins.

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