Retrotransposon-mediated Gene Transfer for Animal Cells

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Abstract Gene delivery methods for animal cells are one of the most important tools in biotechnology fields such as pharmaceutical protein production, generation of transgenic animals and gene therapy. Because retrotransposons can move their own sequences to new genomic locations by a "copy-and-paste" process known as retrotransposition, we attempted to develop a novel gene transfer system based on retrotransposon. A full-length long interspersed element-1 (LINE-1) contains a 5' untranslated region (5'UTR), two non-overlapping open reading frames (ORFs) separated by a short inter-ORF sequence, and a 3'UTR terminating in an adenosine-rich tract. We constructed a LINE-1 vector plasmid including components necessary for retrotransposition. An intron-disrupted *Neo* reporter gene and a scFv-Fc expression unit under the control of CMV promoter were added into 3'UTR in order to evaluate retrotransposition and express scFv-Fc. CHO-K1 cells transfected with the plasmids were screened with G418. The established cell clones produced scFv-Fc proteins in the culture medium. To control retrotransposition steadily, we also established retrotransposon systems that supply ORF2 or ORF1–2 separately. Genomic PCR analysis revealed that transgene sequences derived from the LINE-1 vector were positive in all clones. All the clones tested produced scFv-Fc in the culture medium.

1 Introduction

Gene delivery procedures for animal cells are one of the most important tools in biotechnology fields such as biopharmaceutical protein production, generation of transgenic animals and gene therapy. In order to integrate exogenous genes efficiently into cell chromosomes, various methods represented by viral vectors and recombinases have been developed. We have generated transgenic chickens producing recombinant proteins using retroviral vectors for gene transfer (Kamihira *et al.*, 2005; Kawabe *et al.*, 2012). We have also demonstrated targeted integration of transgenes into Chinese hamster ovary (CHO) cells using the Cre/*loxP* recombination system (Kameyama *et al.*, 2010; Obayashi *et al*., 2012) and established high-producer CHO cells mediated by repeatedly introducing transgene expression units in an enzyme-dependent manner (Wang *et al*., 2017). Recently, genome editing tools using target-designable artificial nucleases such as zinc finger nucleases, TALEN and CRISPR/Cas9 systems have been widely used for genetic engineering of cells. For the production of therapeutic monoclonal antibodies, the genome editing tools have also been applied to CHO cells for genome modification (Lee *et al*., 2015) and transgene knock-in (Inniss *et al*., 2017). By using an efficient knock-in strategy designated precise integration into target chromosome (PITCh) (Nakade *et al*., 2014), we achieved homologous recombination (HR)-independent large gene cassette knock-in for CHO cells using as TALEN- and CRISPR-mediated PITCh system (Sakuma *et al*., 2015).

Retrotransposons are mobile elements that transfer themselves to the cell genome by a "copy-and-paste" mechanism (Elbarbary *et al*., 2016). The process is known as retrotransposition. A retrotransposon, long interspersed element-1 (LINE-1) is categorized as a non-LTR retrotransposon, comprising a 5' untranslated region (5'UTR), two non-overlapping open reading frames (ORFs) (ORF1 and ORF2) separated by a short inter-ORF sequence, and a 3'UTR terminating in an adenosine-rich tract. Although the role of ORF1p remains unknown, ORF2p contains functional enzymes such as reverse-transcriptase (RT) and endonuclease (EN). From the development of LINE-1 retrotransposition assay in the mid-1990s, cell-based LINE-1 functional assays have been essential tools for studying LINE-1 biology (Rangwala and Kazazian Jr., 2009). Figure 1 shows a representative LINE-1 retrotransposition assay based on split neomycin resistant gene (*Neo*), in which an expression cassette of *Neo* reporter gene split by inserting an intron (normal direction) is added into 3'UTR in reverse direction. In this assay, when LINE-1 transcripts are generated

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normally, the intron sequence inserted into *Neo* reporter gene is spliced out to generate the intact form of *Neo* expression cassette, followed by retrotransposition events including reverse transcription and integration into the genome. Thus, retrotransposition is easily assayed by counting G418-resistant colonies. Although the LINE-1 assay has been very useful for studying retrotransposition mechanism, there is no report on the development of LINE-1 vectors for exogenous gene delivery.

Here, we constructed a LINE-1-based vector plasmid for exogenous gene delivery. An intron-disrupted *Neo* reporter gene and an anti-prion single-chain Fv fused with Fc protein (scFv-Fc) expression unit under the control of CMV promoter were inserted into 3'UTR region in order to evaluate retrotransposition and scFv-Fc production. To control retrotransposition, we removed the ORF2 sequence from LINE-1 vector to generate ORF2-deleted LINE-1 vector, and constructed ORF2 expression vectors for providing ORF2 proteins. Using the LINE-1 based vectors, recombinant CHO cells were generated. Transgene copy number and scFv-Fc productivity were evaluated for CHO cell clones.

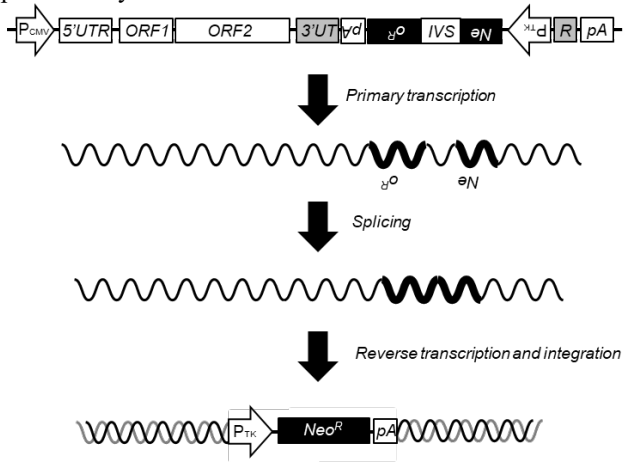

Figure 1. L1 retrotransposition assay.

2 Materials and Methods

2.1 Cell and media

CHO-K1 (RIKEN, Tsukuba, Japan) and recombinant CHO-K1 cells were cultured in Ham's 12 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaillé, France), antibiotics (penicillin and streptomycin, Fujifilm Wako Pure Chemical Industries, Osaka, Japan) in tissue-culture dishes or plates (Thermo Fisher Scientific, Waltham, USA). Cells were cultured at 37°C in a 5% (v/v) CO_2 incubator.

2.2 Vector construction

Mouse LINE-1 element sequences including 5'UTR, ORF1, ORF2 and 3'UTR, were chemically synthesized by Genewiz (South Plainfield, NJ, USA). The sequences were inserted into *Nhe*I- and *Not*I-digested pCEP4 (Invitrogen, Waltham, MA, USA). A *Neo* reporter gene which is split by intron (template strand) was added into 3'UTR together with promoter sequence in reverse direction. A scFv-Fc expression unit under the control of CMV promoter was placed downstream of the *Neo* reporter.

An EBNA-1/OriP sequence was deleted from pCEP4/mL12-sc to generate pC4/mL12-sc (L1) (Figure 2A). To control retrotransposition, the ORF2 sequence was removed from pC4/mL12-sc to generate pC4/mL1-sc (L1[Δorf2], Figure 2B). The ORF2 expression vectors were constructed by introducing ORF2 or ORF1–ORF2 sequences into pZeo/IRES-DsRed to generate pORF2 or pORF1–2, respectively (Figure 2C and D).

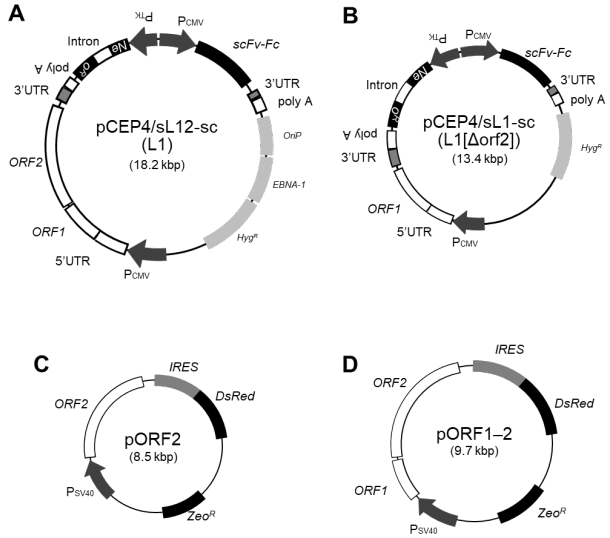

Figure 2. Construction of LINE-1 vectors. (A) Intact LINE-1 vector, pC4/mL12-sc. (B) LINE-1 vector deleted ORF2, pC4/mL1-sc. (C) ORF2 expression vector, pORF2. (D) ORF1−ORF2 expression vector, pORF1–2.

2.3 Retrotransposition

LINE-1 vectors and ORF expression vectors were introduced into CHO-K1 cells using Neon® Transfection System (Invitrogen). The transfection condition was described previously (Kawabe *et al.*, 2017), except for the use of 100 µL kit. Five days after transfection, cells were re-seeded into wells of 6-well plates at the density of 2.0×10^4 cells/well, and drug screening was performed in 2.0 mL medium containing 400 mg/L of G418 (Sigma-Aldrich). The colonies formed after 7 day-culture for selection were washed with phosphate buffered saline solution (PBS), fixed with 4% paraformaldehyde at 37°C for 15 min, and stained with 0.05% crystal violet for 1–5 min to measure the number of cell colonies. Clones were isolated by the colony picking method. Picked clones were subjected to further experiments.

2.4 Copy number and productivity

Genomic DNA extracted from clones using genome extraction kit (MagExtractor -genome-, Toyobo, Osaka, Japan) was subjected to PCR to evaluate

retrotransposition. Copy number of transgene was analyzed by Taqman probe-based real-time PCR (Kawabe *et al.*, 2017). The scFv-Fc production rate was measured as described previously (Kamihira *et al.*, 2005). Briefly, cells were seeded at the density of 2.5×10^4 cells/well in 24-well plates and cultured for 4 days. Viable cell density was determined by the trypan blue exclusion method. The scFv-Fc concentration in culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). The scFv-Fc productivity for each clone was calculated from cell number and scFv-Fc concentration.

3 Results

3.1 Retrotransposition using LINE-1 vector harboring transgene

To evaluate gene transfer based on retrotransposition using the LINE-1 vector harboring a scFv-Fc expression unit (L1), we transfected L1 vector into CHO cells by electroporation. After transfected cells were cultured in the presence of G418, the number of G418-resistant colonies was counted (36.6/20,000) (Figure 3). Genomic DNA extracted from established clones was subjected to PCR using various primer pairs to assay for retrotransposition in genomic structure. Especially, the amplified band using primer pairs for *Neo* is expected to be a spliced size (211 bp) after retrotransposition. DNA fragments with expected size were amplified using specific Neo primers, α and β for all the clones established. Amplicons for *scFv-Fc* transgene were observed for all the clones using primer pair, γ and δ (Figure 4A). These results indicated that transgenes in the LINE-1 vector were introduced into the CHO cell genome by retrotransposition.

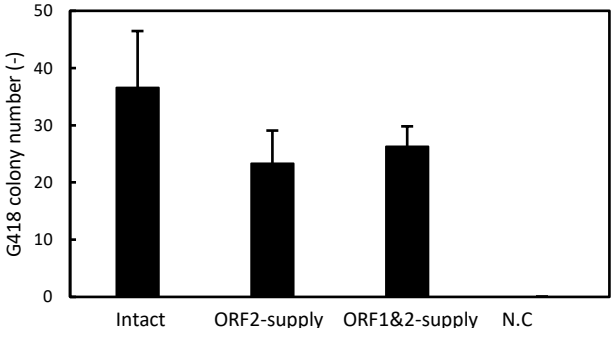

Figure 3. Efficiency of retrotransposition in each condition

3.2 Separated supply system to control retrotransposition

Next, to regulate retrotransposition of LINE-1 vector, we removed *ORF2* gene from LINE-1 vector to generate L1[Δorf2], while ORF2 expression vector (pORF2 or pORF1–2) was used as a helper plasmid. Original LINE-1 encodes for 2 proteins translated from a single RNA transcript containing two non-overlapping *ORF*s (*ORF1* and *ORF2*, and ORF1 protein cooperatively works with ORF2 protein in retrotransposition. Therefore, an expression vector for ORF1–ORF2 was also prepared (pORF1–2).

Retrotransposition was evaluated using three conditions in vector combinations (Intact type (L1), ORF2-supply system (L1[Δorf2] and pORF2) and ORF1–2-supply system (L1[Δorf2] and pORF1–2)). After G418 screening, the numbers of G418-resistant colonies were 23.3 and 26.3 for ORF2- and ORF1–2-supply systems, respectively, under the optimal ratio of vectors, when transfected cells were seeded at the density of 20,000 cells per well (Figure 3). In contrast, no colony was formed in the absence of ORF2 helper vector. These results indicated that there was no significant decrease in colony forming efficiency even after deletion of ORF2 sequence in the LINE-1 vector.

Clones were randomly picked up and subjected to genomic PCR using primer pairs, α and β, γ and δ, as described above. The expected DNA fragments in each region were amplified for the established clones (Figure 4b). Thus, tranagene integration using the LINE-1 vector with ORF2 deletion can be regulated by ORF2 expression.

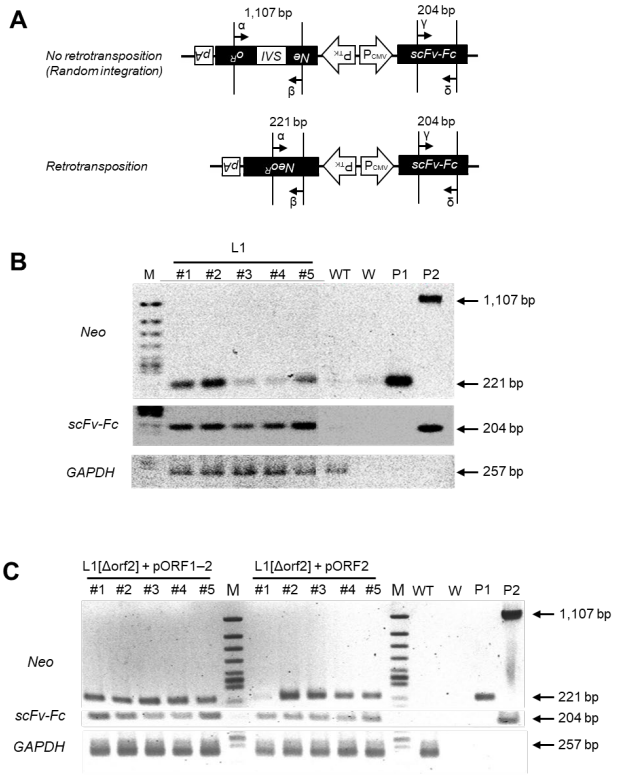

Figure 4. Detection of LINE1-mediated retrotransposition by genomic PCR. (A) Primer design for genomic PCR. (B) Genomic PCR for the clones established using L1 (intact) vector. (C) Genomic PCR for the clones established using L1[Δorf2] and pORF2 or pORF1–2 vectors. Water (W) and wild-type CHO-K1 genome (WT) as negative control. Neo expression vector (pIRES-DsRedExpress (Invitrogen, Waltham, MA, USA), P1) and L1 vector (P2) as positive control. Molecular weight markers (M).

3.3 Productivity of scFv-Fc and copy number of transgene

For 10 clones established from each system, copy number of transgene was evaluated by real-time PCR. Quantitative PCR revealed that scFv-Fc gene was integrated into the genome at the range from one to six copies in the clones. All clones established from each system produced scFv-Fc into culture supernatant, and the productivity exhibited around 0.14 pg/(cell•day) or less.

4 Discussion

The most abundant mobile elements in mammals are non-LTR retrotransposons. Among retrotransposons, LINE-1 sequences are present at –900,000 sites in the human genome, and its content accounts for 21% of the total human genome (Richardson *et al*., 2015). In this study, we developed a gene transfer method based on retrotransposon LINE-1. To control retrotransposition, a system using ORF2-deleted LINE-1 vector and ORF2 expression vector was effective. Transgenes incorporated into the LINE-1 vector could be integrated into the CHO cell genome by retrotransposition.

In this study, the retrotransposition efficiency was at the range from 0.13% to 0.18% in the three systems of L1 vectors. Low-molecular-weight drugs and chemicals may enhance retrotransposition efficiency. It was reported that LINE-1 retrotransposition was promoted for cultured animal cells in the presence of recreational drugs such as methamphetamine (METH) and cocaine, environmental pollutants such as heavy metals (mercury (HgS), cadmium (CdS), and nickel (NiO)) and carcinogens (Goodier, 2016). Ellis *et al*. reported that lentiviral vector production could be enhanced by adding caffeine at a final concentration of 2 to 4 mM (Ellis *et al*., 2011). We also reported that caffeine enhanced the production of retroviral vectors (Kawabe *et al*., 2017). In fact, retrotransposition efficiency enhanced 1.8-fold in the presence of a suitable concentration of caffeine at transfection of L1 vector (data not shown). Caffeine is not a harmful agent at low concentration and the method is simple and inexpensive.

LINE-1 retrotransposition assay can simply distinguish between retrotransposon-mediated gene integration and random integration, and it has been used to analyze the mechanism of retrotransposition. However, the transcription interference may be caused because transcriptional orientations are opposite to each other between transcriptions of retrotransposon and *Neo* reporter gene (Shearwin *et al*., 2005). Vector design to eliminate transcription interference will be necessary to increase the retrotransposition efficiency.

In this study, the LINE-1-based vectors delivered 1 to 6 copies of transgenes into the CHO-K1 cell genome and the cells produced with 0.01 to 0.14 pg of scFv-Fc per cell per day. The productivity is not high in comparison with other gene transfer method for CHO-K1 cells (Wang *et al.*, 2017). Transgene expression may affect the surrounding enviroment of integrated locus of genome structure, leading to gene silencing in some situations (Pannell and Ellis, 2001). Targeted transgene integration into a specific locus provides more predictable gene expression and less clonal variability. Therefore, the combination of genome editing tools and engineered LINE-1 ORFs may be effective for realizing targeted integration using retrotransposon-based vectors.

In conclusion, we developed a novel gene transfer procedure using LINE-1 retrotransposon vector. By separating ORF2 gene from LINE-1 vector, retrotransposition could be controlled by providing ORF2 expression from a helper vector encoding ORF2 expression unit. Recombinant antibody gene was integrated into the CHO cell gemone using the LINE-1 vector and antibody was produced from the cells. This method will provide a novel gene delivery tool for modifying cell genomes.

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