Cre/Lox-based RMCE for efficient gene-of-interest exchange in CHO cells

Jaewon Kim [1] and Joon Tae Park[1]

[1]Incheon National University

July 29, 2020

Abstract

Traditional cell line development is based on random genomic integration of transgenes. Random integration leads to unpredictable expression and results in clonal heterogeneity requiring a tedious screening procedure. Therefore, a new strategy is needed to establish clones that exhibit stable transgene expression. Here, we performed CRISPR/Cas9-mediated site-specific integration (SSI) to incorporate a landing pad (LP; containing mCherry) at a genomic hotspot (Fer1L4) allowing stable and strong expression. Site-specific integration of LP on Fer1L4 was demonstrated by sequencing results representing the swapped sequences in mCherry-expressing cells. We then performed Cre/Lox-based recombinase-mediated cassette exchange (RMCE) to exchange LP with a targeting vector (TV; containing GFP) in clones established by CRISPR/Cas9-mediated SSI. The success of Cre/Lox-based RMCE was evidenced by sequencing results representing the swapped sequences in GFP-expressing cells. Furthermore, the swapped clones expressing GFP was enriched up to 80%, indicating that the efficiency of Cre/Lox-based RMCE would be sufficient to swap pre-existing cassettes with gene-of-interest (GOI). Taken together, our study provides a new platform for Cre/Lox-based RMCE to iteratively establish stable clones from existing ones previously established by SSI at a genomic hotspot.

Introduction

Biopharmaceuticals have revolutionized the treatment of a wide range of diseases, and are increasingly being used in medicines (Kesik-Brodacka, 2018). Chinese hamster ovary (CHO) cells have been the most common hosts for the production of therapeutic proteins (Lai, Yang, & Ng, 2013). Traditional cell line development in CHO cells is based on random integration of recombinant constructs into the genome, resulting in clones with variable expression (Lai et al., 2013). Heterologous expression within cell pools requires the selection of multiple clones and then following a tedious screening procedure (Li, Vijayasankaran, Shen, Kiss, & Amanullah, 2010). Moreover, due to lack of control over the genome integration site, protein productivity of selected clones may decrease over time, leading to clonal heterogeneity (Coates et al., 2005). To overcome such possible shortcomings, site-specific integration (SSI) has been proposed for use in cell line development (N. K. Hamaker & K. H. Lee, 2018). SSI integrates gene-of-interest (GOI) into a predetermined genomic hotspot to enable stable and robust transgene expression. This makes it possible to generate isogenic clones with consistent productivity and stability, consequently reducing clone screening and selection time (N. K. Hamaker & K. H. Lee, 2018).

Recombinase-mediated cassette exchange (RMCE) is a procedure in reverse genetics allowing precise integration of a genomic target cassette (Turan, Zehe, Kuehle, Qiao, & Bode, 2013). Specifically, it allows double-reciprocal crossover events between two pairs of heterospecific recombinase target sites using a site-specific recombinase including Tyr-recombinases (e.g. Cre or Flp) and Ser-recombinases (e.g. ΦC31 or Bxb1) (Turan et al., 2013).Thus, RMCE exchanges only a preexisting gene cassette with an analogous one carrying GOI without changing cellular characteristics (Turan et al., 2013). It is the most advanced platform to establish new clones with similar productivity and characteristics compared to existing clones (Turan et al.,

2013). Cre/Lox-based RMCE functions exceptionally well, exhibiting cassette exchange events with high fidelity (Gidoni, Srivastava, & Carmi, 2008; Schnütgen, Stewart, von Melchner, & Anastassiadis, 2006). Cre recombinase allows for repetitive cumulative gene targeting to predefined genomic loci in mammalian cells using different target sites such as Lox5171 and LoxP (Gidoni et al., 2008; Schnütgen et al., 2006).

In this study, we aimed to evaluate whether the Cre/Lox-based RMCE constitutes an effective strategy to acquire clones from existing ones established by CRISPR/Cas9-mediated SSI at a genomic hotspot allowing stable expression. Here, we report that Cre/Lox-based RMCE generates new clones by exchanging only a existing cassette with a targeting cassette carrying GOI. Our results will open up a new paradigm of commercial protein production by repeatedly establishing cell lines with predictable productivity and properties.

Materials and Methods

Plasmid design and construction

Standard cloning techniques were used to construct a recombinant plasmid. LP was designed where Lox5171/LoxP sites flanked mCherry followed by CMV promotor-driven thymidine kinase (*TK*) gene (Fig. 1A). The LP also contained the 5' homologous arm (HA) (0.2 kb region of exon 1 in *Fer1L4* present before the transcription start site) and 3' HA (0.2kb region of exon 1 in *Fer1L4* present after the transcription start site) (Fig. 1A). TV was designed where Lox5171/LoxP sites flanked GFP followed by SV40 promoter-driven neomycin resistance gene (Fig. 1B). Cre recombinase expressing plasmid is composed of SV40 promoter-driven *Cre* gene.

Cell culture

CHO DG44 cell line (A1100001; Thermo Fisher Scientific, Waltham, MA, USA) was maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose (SH30243.01; Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (SH30919.03; Hyclone), 10 mM sodium hypoxanthine and 1.6 mM thymidine (2068642; Gibco, Waltham, MA, USA) along with 100 U/ml penicillin and 100 μg/ml streptomycin (SV30079.01; Hyclone). Cells were cultured in ambient air (20% O_2) supplemented with 5% CO_2. For batch culture, cells were cultivated in a 250 ml Erlenmeyer flask (73250; SPL, Pocheon, Korea) with a working volume of 30 ml. Cells were incubated with 150 rpm agitation, and passaged every 3 d.

CRISPR/Cas9-mediated site specific integration of LP on*Fer1L4*

Fer1L4 single guide (sg) RNA sequence (5′-AGACGCCTAACAGAGCTGCCAGG-3′) was designed using the CasFinder system (Aach, Mali, & Church, 2014) and obtained from ToolGen (Seoul, Korea). 2×10^6 cells were transfected with 2 μg of LP DNA, 4 μg of Cas9 protein and 1 μg sgRNA using SG cell line 4D-nucleofector® X Kit L (V4XC-3024; Lonza, Basel, Switzerland). Transfected cells were selected for 2 weeks with 50 μM blasticisdin (ant-bl-05; Invitrogen, Waltham, MA, USA). During selection, the medium was changed every 4 d. After selection, single cells were seeded per well in 150 μl medium in 96-well plates (353072; Falcon) and incubated for 15 d. Cells expressing mCherry were screened using fluorescence microscopy (Axiovert 200, Carl Zeiss, Oberkochen, Germeny).

Preparation of genomic DNA (gDNA) and complementary DNA (cDNA)

gDNA was prepared from 2×10^6 cells using Solg genomic DNA prep kit (SGD41-C100; Solgent, Daejon, Korea) according to the manufacturer's instructions. mRNA was isolated from 2 x 10^6 cells using Rnasy Mini Kit (74104; QIAGEN, Hilden, Germany). mRNA was reverse-transcribed using phusion RT-PCR Kit (F-546S; Thermo Fisher Scientific) according to the manufacturer's instructions. Purity and concentration of the gDNA and cDNA were determined using a DS-11 Spectrophotometer (DeNovix, Wilmington, DE, USA).

Nested PCR and sequencing

All primers were designed using the Snap Gene software (GSL Biotech LLC, Chicago, IL, USA) and synthesized by Macrogen (Seoul, Korea). Nested PCR was performed using a thermal cycler (9700; Perkin Elmer, Waltham, MA, USA) using Solg 2xTaq PCR smart mix 2 (STD02-M50h; Solgent). Nested PCR was performed with 20 ng of gDNA, with denaturation at 95degC for 5 min followed by 40 cycles of 94degC for 30 s, 57degC for 30 s, and 70degC for 10 s. PCR fragments were purified using Solg gel and PCR purification system (SGP04-C200; Solgent) and sequenced by Macrogen.

Quantitative PCR (qPCR)

qPCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a Solg 2x real-time PCR smart mix (SRH83-M40h; Solgent). RT-qPCR was performed either with 20 ng of gDNA or cDNA, with denaturation at 95degC for 5 min followed by 40 cycles of 94degC for 30 s, 57degC for 30 s, and 70degC for 10 s.

RMCE-mediated generation of stable cell lines

For RMCE, 2 x 10^6 cells were transfected with 2 μg of TV and 2 μg of Cre expression vector using SG Cell Line 4D-Nucleofector® X Kit L (V4XC-3024; Lonza). Transfected cells were selected for 2 weeks with 500 μg/mL G418 (ant-gn-1; Invitrogen). During selection, the medium was changed every 4 d. After selection, the percentage of cells expressing GFP was measured on a Gallios flow cytometer platform (Beckman Coulter, Brea, CA, USA). Enrichment of GFP-positive cells was performed using a BD FACSAria cell sorter equipped with a 488 nm laser. Sorted cells were seeded 1 cell per well in 150 μl medium in 96-well plates, and incubated for 15 da. Cells expressing GFP were screened using a fluorescence microscopy (Axiovert 200; Carl Zeiss).

Statistical analyses

Statistical analyses were performed using a standard statistical software package (SigmaPlot 12.5; Systat Software, San Jose, CA, USA). Mann-Whitney *U* tests were used to determine whether differences were statistically significant.

Results

CRISPR/Cas9-mediated site specific integration of LP on*Fer1L4*

Genomic hotspot is a locus that allows for stable and strong transgene expression (Nathaniel K. Hamaker & Kelvin H. Lee, 2018). *Fer1L4* has been identified as genomic hotspot in CHO cells (Zhang et al., 2015). Hence, we selected this region as a targeted integration site. LP was designed where Lox5171/LoxP sites flanked mCherry followed by CMV promotor-driven thymidine kinase (*TK*) gene (Fig. 1A). It also contained the 5' HA (0.2 kb region of *Fer1L4* exon 1 present before the transcription start site) and 3' HA (0.2kb region of *Fer1L4* exon 1 present after the transcription start site) (Fig. 1A). For RMCE-mediated gene swapping, TV was designed where Lox5171/LoxP sites flanked GFP, followed by an SV40 promoter-driven neomycin resistance gene (Fig. 1B).

We then performed CRISPR/Cas9-mediated homologous recombination to integrate LP into the *Fer1L4* exon 1 region (Fig. 2A). Fourteen days after drug selection, single cell clones were isolated using limiting dilution in 96-well plates, and 12 clones expressing mCherry were seleced (Fig. 2B). We then examined how many copies of*Fer1L4* were targeted by LP. A *Fer1L4* forward (Fwd) primer was designed to bind to the sequence before the 5' HA, and a*Fer1L4* reverse (Rev) primer was designed to bind to the sequence after the 3' HA (Fig. 2C). When *Fer1L4* is not targeted by LP, a 200 bp PCR product will be amplified (Fig. 2C). However, when*Fer1L4* is targeted by LP, a 3,300 bp PCR product will be amplified (Fig. 2C). Therefore, to amplify only the *Fer1L4* not targeted by LP, qPCR was performed with a 5 s extension time. Genomic DNA from CHO cells was used as a negative control (Fig. 2D). Eight of the 12 clones showed half the value compared to the negative control, indicating that only one of the two copies of *Fer1L4* was targeted by LP (Fig. 2D; arrowhead).

We then examined the nucleotide sequence of the recombinant region. To amply the *Fer1L4* region targeted by LP, nested PCR was performed on genomic DNA with 3 sets of primers (Fig. 3A). The 1st Fwd primer

was designed to bind to the sequence before the 5' HA, and the 1st Rev primer was designed to bind to the mCherry sequence (Fig. 3A). The 1st PCR amplification using the 1st primer set generated a template for a 2nd PCR (Fig. 3A and B). Subsequently, the 2nd PCR amplification with the 2nd primer set generated a template for a 3rd PCR (Fig. 3A and B). Finally, the 3rd PCR amplification using the 3rdset of primers generated a 348 bp amplicon in clone #11 (Fig. 3B; red arrow). However, other clones (clones #4, #6, #8 and #9) were found to have undergone LP integration in *Fer1L4,* but did not yield the 348 bp amplicon (Fig. 3B). These results indicated that the quality of genomic DNA extracted from those clones was not sufficient to generate PCR amplicons. However, further verification is needed to clarify this possibility. We then performed DNA sequencing on the amplified PCR products from clone #11, and found that the sequence of the PCR product was identical to that of the *Fer1L4* region targeted by LP. This result suggests site-specific LP targeting in *Fer1L4* (Fig. 3C).

Cre/Lox-based RMCE for efficient GOI exchange in CHO cells

Cre recombinase allows double recombination events between two pairs of heterologous Lox sites, allowing LP to be exchanged with TV (Saraf-Levy et al., 2006). To perform Cre/Lox-mediated RMCE, 2 μg of TV DNA was co-transfected with 2 μg of Cre expression vector in clones targeted by LP (Fig. 4A). Since TV has no promoter, GFP will be expressed only when LP is correctly exchanged with TV (Fig. 4A). Therefore, in order to select the successfully exchanged clones, cells expressing GFP were identified during the single cell cloning procedure. All established clones expressed GFP but not mCherry, indicating RMCE-mediated mCherry-to-GFP swapping (Fig.4B).

We then examined the nucleotide sequence of the swapped region. PCR was performed on genomic DNA with primer sets before and after Lox5171 (Fig. 4C). A band corresponding to the expected size appeared in GFP-positive clones obtained after RMCE, whereas the PCR band did not appear in clones obtained before RMCE (Fig. 4C; red arrow). Subsequent DNA sequencing of the purified PCR product revealed that the sequence was identical to that of the mCherry-to-GFP swapped *Fer1L4* region (Fig. 4C).

The success of the Cre/Lox-based RMCE system allowed us to investigate the mCherry-to-GFP swap rate by evaluating the percentage of cells expressing only GFP, without expressing mCherry (Fig. 4D). Before RMCE, the percentage of such cells was 0.5%, indicating that a small proportion of clones targeted by LP exhibit autofluorescence that can be detected in the GFP filter (Fig. 4D). After RMCE, the percentage of these cells significantly increased to 2.95%, indicating that the Cre/Lox-based RMCE system successfully functioned in CHO cells (Fig. 4D).

Establishment of optimal conditions for RMCE

The ratio between TV and Cre recombinase is a decisive factor in RMCE (Du, Hu, Ayala, Sauer, & Zhang, 2009). Thus, to improve RMCE efficiency, we investigated the optimal ratio between TV and Cre recombinase. To achieve this, 2 μg of TV DNA was co-transfected with various amounts of Cre expression vector in clones targeted by LP. Further, 2 μg TV with 0.5 μg Cre expression vector significantly increased the percentage of cells expressing GFP compared to the initial conditions (2 μg TV DNA with 2 μg Cre expression vector) (Fig. 5A). These results indicated that a 4:1 ratio involving TV (2 μg) and Cre expression vector (0.5 μg) was the optimal condition for RMCE (Fig. 5A).

The application of multi-cistronic gene cassettes capable of simultaneously expressing GOI and GFP in TV may be a breakthrough for RMCE; GOI can be used for protein production, and GFP can be used as a sorting marker for selecting swapped clones (Bouabe, Fässler, & Heesemann, 2008). Thus, we performed FACS sorting to enrich GFP-positive clones established after the identification of the optimal RMCE conditions. FACS sorting enhanced the percentage of GFP-positive cells from 3.94% up to 80%, indicating that RMCE efficiency can be indirectly increased by enriching GFP-positive cells capable of simultaneously expressing GOI (Fig. 5B).

Discussion

The current focus in the biopharmaceutical industry is to identify cells that exhibit predictable expression

levels with the expected amount of protein (Wang et al., 2018). However, traditional procedures for cell line development are based on random transgene integration into the genome (Lai et al., 2013). Thus, the crosstalk between cis- and trans-acting factors in the neighboring chromosome influences whether transgenes are silent or active (West & Fraser, 2005). Even after establishing single cell clones, silencer sequences present on adjacent chromosomes contribute to the spread of condensed chromatin to the transgene, leading to transgene silencing (Wang et al., 2018). To circumvent possible positional effect, the use of SSI has been proposed (Lee, Kallehauge, Pedersen, & Kildegaard, 2015). Targeted integration of transgenes into predefined chromosomal locations leads to more predictable and stable transgene expression (Lee et al., 2015). Recently, SSI has been further improved using RMCE, a technology that exchanges existing genetic cassettes established by SSI with similar cassettes carrying GOI (Turan et al., 2011). RMCE allows re-establishment of new clones carrying new GOI from preexisting clones carrying different GOI (Turan et al., 2013). Clones that established via RMCE behave similarly to the preexisting clones in terms of productivity, cell growth and metabolism (Turan et al., 2013) (Fig. 6). In this study, we established Cre/Lox-based RMCE for efficient GOI exchange in CHO cells. CRISPR/Cas9-mediated SSI integrated LP into a genomic hotspot region (*Fer1L4*). Cre/Lox-mediated RMCE replaced LP with TV, as evidenced by DNA sequencing results showing the recombinant region in*Fer1L4* . Furthermore, optimal conditions for RMCE, which significantly improved RMCE efficiency, were established. To our knowledge, this study provides the first demonstration that Cre/Lox-based RMCE can reestablish a derivative clone from an existing one previously established by SSI. As the cellular characteristics and productivity of clones regenerated by RMCE were as expected and the variation between clones was minimal (Turan et al., 2013), our results can be actively used in establishing new clones with predictable productivity from preexisting clones previously established by SSI (Fig. 6).

Genomic hotspots refers to genomic loci that confer exceptional stability and enhanced transcriptional activity (Nathaniel K. Hamaker & Kelvin H. Lee, 2018). Thus, targeting GOI in such genomic hotspots is prerequisite process for SSI-based cell line development (Nathaniel K. Hamaker & Kelvin H. Lee, 2018). The importance of genomic hotspots in SSI-based cell line development is supported by the fact that it enables antibody production regardless of antibody format (Crawford et al., 2013; Zhang et al., 2015). Genomic hotspots can guarantee higher protein productivity, but are limited to include up to two copies of LPs (Gaidukov et al., 2018). Therefore, a strategy of integrating multiple copies of LPs would increase the protein productivity in clones established by SSI-based RMCE. Thus, we propose the application of a multi-cistronic gene vector using the 2A system to produce two or more independent proteins at the stoichiometric level (Donnelly, Hughes, et al., 2001; Donnelly, Luke, et al., 2001). Up to 9 proteins linked to 2A sequence have been co-translated at equivalent levels (Geier, Fauland, Vogl, & Glieder, 2015). Thus, multi-cistronic $(GOI\text{-}2A)^8$-GFP cassettes can be used to simultaneously express GFP as a sorting marker, and GOI for protein production (Fig. 7). The exchanged clones expressing GFP in this study were concentrated to 80% through FACS sorting, so that the application of multi-cistronic gene cassettes would help establish new clones via RMCE. Furthermore, multiple GOI linked to 2A can generate GOI at the stoichiometric level. Hence, the application of a multi-cistronic gene cassette can solve the shortcomings of SSI-based RMCEs that incorporate up to two copies of GOI (Fig. 7). Taken together, the possible disadvantages of SSI-based RMCEs can be solved by using multi-cistronic gene cassettes on TVs, as new clones can be easily obtained through RMCE and show an improvement in GOI production.

In summary, we performed SSI to target LP incorporation into a genomic hotspot, and established a Cre/Lox-based RMCE system to exchange LP with TV. This system efficiently re-establish new clones from existing ones by only exchanging a preexisting gene cassette for an analogous cassette carrying GOI. RMCE efficiency was indirectly improved by up to 80% when FACS sorting was performed after RMCE. Therefore, our results provide evidence that Cre/Lox-based RMCE system will be a useful strategy for iteratively establishing clones that confer predictable productivity and properties.

Acknowledgments

This research was supported by a Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning (NRF-

2018R1D1A1B07040293). This research was also supported by Research Assistance Program (2019) in the Incheon National University.

Author Contributions

KJW and JTP conceived of and designed the experiments. KJW performed the experiments. KJW, and JTP analyzed the data. KJW and JTP wrote and edited the paper.

Conflict of interest

The authors declare no competing financial interests.

References

Aach, J., Mali, P., & Church, G. M. (2014). CasFinder: Flexible algorithm for identifying specific Cas9 targets in genomes. *bioRxiv* , 005074. doi:10.1101/005074

Bouabe, H., Fässler, R., & Heesemann, J. (2008). Improvement of reporter activity by IRES-mediated polycistronic reporter system. *Nucleic acids research, 36* (5), e28-e28. doi:10.1093/nar/gkm1119

Coates, C. J., Kaminski, J. M., Summers, J. B., Segal, D. J., Miller, A. D., & Kolb, A. F. (2005). Site-directed genome modification: derivatives of DNA-modifying enzymes as targeting tools. *Trends in Biotechnology, 23* (8), 407-419. doi:*https://doi.org/10.1016/j.tibtech.2005.06.009*

Crawford, Y., Zhou, M., Hu, Z., Joly, J., Snedecor, B., Shen, A., & Gao, A. (2013). Fast identification of reliable hosts for targeted cell line development from a limited-genome screening using combined φC31 integrase and CRE-Lox technologies.*Biotechnol Prog, 29* (5), 1307-1315. doi:10.1002/btpr.1783

Donnelly, M. L. L., Hughes, L. E., Luke, G., Mendoza, H., Ten Dam, E., Gani, D., & Ryan, M. D. (2001). The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. *J Gen Virol, 82* (Pt 5), 1027-1041. doi:10.1099/0022-1317-82-5-1027

Donnelly, M. L. L., Luke, G., Mehrotra, A., Li, X., Hughes, L. E., Gani, D., & Ryan, M. D. (2001). Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *J Gen Virol, 82* (Pt 5), 1013-1025. doi:10.1099/0022-1317-82-5-1013

Du, Z. W., Hu, B. Y., Ayala, M., Sauer, B., & Zhang, S. C. (2009). Cre recombination-mediated cassette exchange for building versatile transgenic human embryonic stem cells lines. *Stem Cells, 27* (5), 1032-1041. doi:10.1002/stem.38

Gaidukov, L., Wroblewska, L., Teague, B., Nelson, T., Zhang, X., Liu, Y., . . . Weiss, R. (2018). A multi-landing pad DNA integration platform for mammalian cell engineering. *Nucleic acids research, 46* (8), 4072-4086. doi:10.1093/nar/gky216

Geier, M., Fauland, P., Vogl, T., & Glieder, A. (2015). Compact multi-enzyme pathways in P. pastoris.*Chem Commun (Camb), 51* (9), 1643-1646. doi:10.1039/c4cc08502g

Gidoni, D., Srivastava, V., & Carmi, N. (2008). Site-specific excisional recombination strategies for elimination of undesirable transgenes from crop plants. *In Vitro Cellular & Developmental Biology - Plant, 44* (6), 457-467. doi:10.1007/s11627-008-9140-3

Hamaker, N. K., & Lee, K. H. (2018). Site-specific Integration Ushers in a New Era of Precise CHO Cell Line Engineering. *Current opinion in chemical engineering, 22* , 152-160. doi:10.1016/j.coche.2018.09.011

Hamaker, N. K., & Lee, K. H. (2018). Site-specific Integration Ushers in a New Era of Precise CHO Cell Line Engineering. *Current opinion in chemical engineering, 22* , 152-160. doi:10.1016/j.coche.2018.09.011

Kesik-Brodacka, M. (2018). Progress in biopharmaceutical development. *Biotechnology and applied biochemistry, 65* (3), 306-322. doi:10.1002/bab.1617

Lai, T., Yang, Y., & Ng, S. K. (2013). Advances in Mammalian cell line development technologies for recombinant protein production. *Pharmaceuticals (Basel, Switzerland), 6* (5), 579-603. doi:10.3390/ph6050579

Lee, J. S., Kallehauge, T. B., Pedersen, L. E., & Kildegaard, H. F. (2015). Site-specific integration in CHO cells mediated by CRISPR/Cas9 and homology-directed DNA repair pathway. *Sci Rep, 5* (1), 8572. doi:10.1038/srep08572

Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R., & Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. *mAbs, 2* (5), 466-479. doi:10.4161/mabs.2.5.12720

Saraf-Levy, T., Santoro, S. W., Volpin, H., Kushnirsky, T., Eyal, Y., Schultz, P. G., . . . Carmi, N. (2006). Site-specific recombination of asymmetric lox sites mediated by a heterotetrameric Cre recombinase complex. *Bioorg Med Chem, 14* (9), 3081-3089. doi:10.1016/j.bmc.2005.12.016

Schnütgen, F., Stewart, A. F., von Melchner, H., & Anastassiadis, K. (2006). Engineering Embryonic Stem Cells with Recombinase Systems. In *Methods in Enzymology* (Vol. 420, pp. 100-136): Academic Press.

Turan, S., Galla, M., Ernst, E., Qiao, J., Voelkel, C., Schiedlmeier, B., . . . Bode, J. (2011). Recombinase-mediated cassette exchange (RMCE): traditional concepts and current challenges. *J Mol Biol, 407* (2), 193-221. doi:10.1016/j.jmb.2011.01.004

Turan, S., Zehe, C., Kuehle, J., Qiao, J., & Bode, J. (2013). Recombinase-mediated cassette exchange (RMCE) - a rapidly-expanding toolbox for targeted genomic modifications. *Gene, 515* (1), 1-27. doi:10.1016/j.gene.2012.11.016

Wang, B., Albanetti, T., Miro-Quesada, G., Flack, L., Li, L., Klover, J., . . . Hawley-Nelson, P. (2018). High-throughput screening of antibody-expressing CHO clones using an automated shaken deep-well system. *Biotechnol Prog, 34* (6), 1460-1471. doi:10.1002/btpr.2721

West, A. G., & Fraser, P. (2005). Remote control of gene transcription. *Hum Mol Genet, 14 Spec No 1* , R101-111. doi:10.1093/hmg/ddi104

Zhang, L., Inniss, M. C., Han, S., Moffat, M., Jones, H., Zhang, B., . . . Young, R. J. (2015). Recombinase-mediated cassette exchange (RMCE) for monoclonal antibody expression in the commercially relevant CHOK1SV cell line. *Biotechnol Prog, 31* (6), 1645-1656. doi:10.1002/btpr.2175

Figure Legends

Fig. 1. Schematic of LP and TV. (A) LP was designed where Lox5171/LoxP sites flanked mCherry, followed by a CMV promotor-driven thymidine kinase (*TK*) gene. LP also contained the 5' HA (0.2 kb region of*Fer1L4* exon 1 present upstream of the transcription start site) and 3' HA (0.2 kb region of *Fer1L4* exon 1 present after the start site). (B) TV was designed where Lox5171/LoxP sites flanked GFP, followed by an SV40 promoter-driven neomycin resistance gene.

Fig. 2. Targeted integration of LP mediated by CRISPR/Cas9. (A) Schematic diagram of CRISPR/Cas9-mediated SSI of LP in *Fer1L4* region. Only when RMCE LP was incorporated into *Fer1L4* exon 1, mCherry was expressed under the control of the endogenous *Fer1L4* promoter. (B) Differential interference contrast (DIC) and fluorescence microscopy images of single cell clones targeted by LP (red: mCherry, scale bar: 50 μm). (C) Schematic diagram: PCR reaction was performed to determine *Fer1L4* copy number targeted by LP. *Fer1L4* Fwd primer was designed to bind to the sequence before the 5' HA, and *Fer1L4* Rev primer was designed to bind to the sequence after the 3' HA. When *Fer1L4* is not targeted by LP, 200 bp PCR product will be amplified. However, when *Fer1L4* is targeted by LP, 3,300 bp PCR product will be amplified. (D) Comparison of relative *Fer1L4* copy number between a negative control and clones targeted by LP. To amplify only the *Fer1L4* locus not targeted by LP, qPCR was performed with a 5 s extension time. Eight of 12 clones showed half the value compared to the negative control, indicating that only one copy of the *Fer1L4* genomic region was targeted by LP (arrowhead).

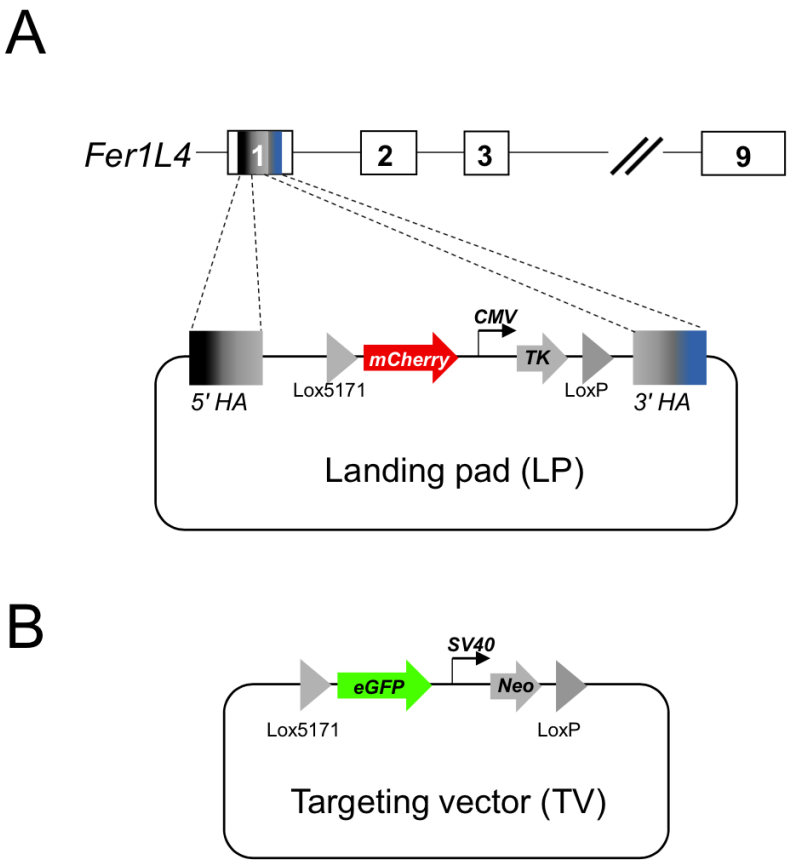
Fig. 3. Verification of recombined sequences in single cell clones targeted by LP. (A) Schematic diagram of nested PCR to amplify the LP-targeted region. The 1st Fwd primer was designed to bind to the sequence before the 5' HA, and the 1st Rev primer was designed to bind to the mCherry sequence. The 1st PCR amplification with 1stprimer set generated the anticipated amplicon (590 bp), which was used as template for the 2nd PCR. Subsequently, the 2nd PCR amplification with 2ndprimer set generated the anticipated amplicon (436 bp), which was used as templates for the 3rd PCR. (B) Picture of agarose gel electrophoresis showing the PCR amplicon generated by the nested PCR. The 3rd PCR amplification with the 3rd primer set generated an amplicon (348 bp) in clone #11 (red arrow). (C) The DNA sequence of the amplified PCR product was identical to that of the *Fer1L4* region targeted by LP.

Fig. 4. Cre/Lox-based RMCE for efficient GOI exchange in CHO cells. (A) Schematic diagram of GOI swapping using Cre/Lox-based RMCE. Cre recombinase allows double recombination between two pairs of heterologous Lox sites, exchanging LP with TV. Since TV has no promoter, GFP will be expressed if LP is correctly exchanged with TV. (B) DIC and fluorescence microscopy images of single cell clones generated by RMCE (red: mCherry, green: GFP, scale bar: 50 μm). The success of these Cre/Lox-based RMCE resulted in mCherry-to-GFP swapping, allowing the expression of GFP rather than mCherry. (C) Schematic diagram of PCR to amplify swapped regions. PCR was performed on genomic DNA with primer sets targeting before and after Lox5171. A band corresponding to the expected size appeared in GFP positive clones, whereas no PCR band appeared in the negative control. The DNA sequence of the amplified PCR product was identical to that of the mCherry-to-GFP swapping region in*Fer1L4* . (D) The mCherry-to-GFP swapping rate was measured by flow cytometry. The percentage of cells expressing GFP without mCherry was calculated before and after RMCE (**$P < 0.01$, Mann-Whitney U test). Means ± S.D., N = 3.

Fig. 5. Establishment of optimal conditions for RMCE. (A) 2 μg of TV DNA was co-transfected with various amounts of Cre expression vector in clones targeted by LP. The percentage of cells expressing GFP, but not mCherry, was measured by flow cytometry. (**$P < 0.01$, Mann-Whitney U test). Means ± S.D., N = 3. (B) FACS sorting-mediated enrichment of GFP-positive cells established under the optimized RMCE conditions. The percentage of cells expressing GFP, but not mCherry, was measured by flow cytometry. (**$P < 0.01$, Mann-Whitney U test). Means ± S.D., N = 3.

Fig. 6. Cre/Lox based RMCE reestablishes new clones that expect similar productivity and properties compared to existing ones.

Fig. 7. Application of multi-cistronic gene cassettes capable of simultaneously expressing GOI and GFP in TV. Multiple GOI linked to 2A can be used for protein production and GFP can be used as a sorting marker for selecting swapped clones.



Hosted file

FIGURE.pdf available at https://authorea.com/users/347080/articles/472906-cre-lox-based-rmce-for-efficient-gene-of-interest-exchange-in-cho-cells