

# Streamlined Human Cell-Based Recombinase-Mediated Cassette Exchange Platform Enables Multigene Expression for the Production of Therapeutic Proteins

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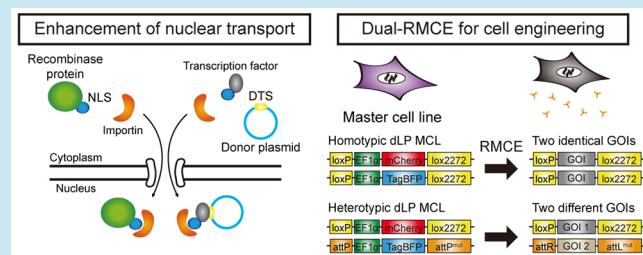
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**ABSTRACT:** A platform, based on targeted integration of transgenes using recombinase-mediated cassette exchange (RMCE) coupled with CRISPR/Cas9, is increasingly being used for the development of mammalian cell lines that produce therapeutic proteins, because of reduced clonal variation and predictable transgene expression. However, low efficiency of the RMCE process has hampered its application in multicopy or multisite integration of transgenes. To improve RMCE efficiency, nuclear transport of RMCE components such as site-specific recombinase and donor plasmid was accelerated by incorporation of nuclear localization signal and DNA nuclear-targeting sequence, respectively. Consequently, the efficiency of RMCE in dual-landing pad human embryonic kidney 293 (HEK293) cell lines harboring identical or orthogonal pairs of recombination sites at two well-known human safe harbors (AAVS1 and ROSA26 loci), increased 6.7- and 8.1-fold, respectively. This platform with enhanced RMCE efficiency enabled simultaneous integration of transgenes at the two sites using a single transfection without performing selection and enrichment processes. The use of a homotypic dual-landing pad HEK293 cell line capable of incorporating the same transgenes at two sites resulted in a 2-fold increase in the transgene expression level compared to a single-landing pad HEK293 cell line. In addition, the use of a heterotypic dual-landing pad HEK293 cell line, which can incorporate transgenes for a recombinant protein at one site and an effector transgene for cell engineering at another site, increased recombinant protein production. Overall, a streamlined RMCE platform can be a versatile tool for mammalian cell line development by facilitating multigene expression at genomic safe harbors.

**KEYWORDS:** targeted integration, recombinase-mediated cassette exchange, nuclear transport, human cell engineering, multigene expression, therapeutic proteins



For the development of mammalian cell lines that produce therapeutic proteins, targeted integration of the gene of interest (GOI) is increasingly being used to mitigate clonal variation and reduce the cost of cell line development.<sup>1,2</sup> For targeted integration, two approaches such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas9) and recombinase-mediated cassette exchange (RMCE) have been attempted in Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) 293 cells—two major workhorses for the production of therapeutic proteins.<sup>3–6</sup> In the CRISPR/Cas9 system, guide RNA (gRNA)-Cas9 complex induces DNA double strand breaks at a target site and a donor template with 5' and 3' homologous sequences can be integrated by homology-directed repair.<sup>7</sup> Site-specific recombination mediated by recombinase induces rearrangement of genome sequence, such as excision, inversion, integration, and cassette exchange, which is dependent on the number, position, and orientation of recombination sites.<sup>8</sup> Unlike CRISPR/Cas9-mediated integration that depends on the host DNA repair system, RMCE

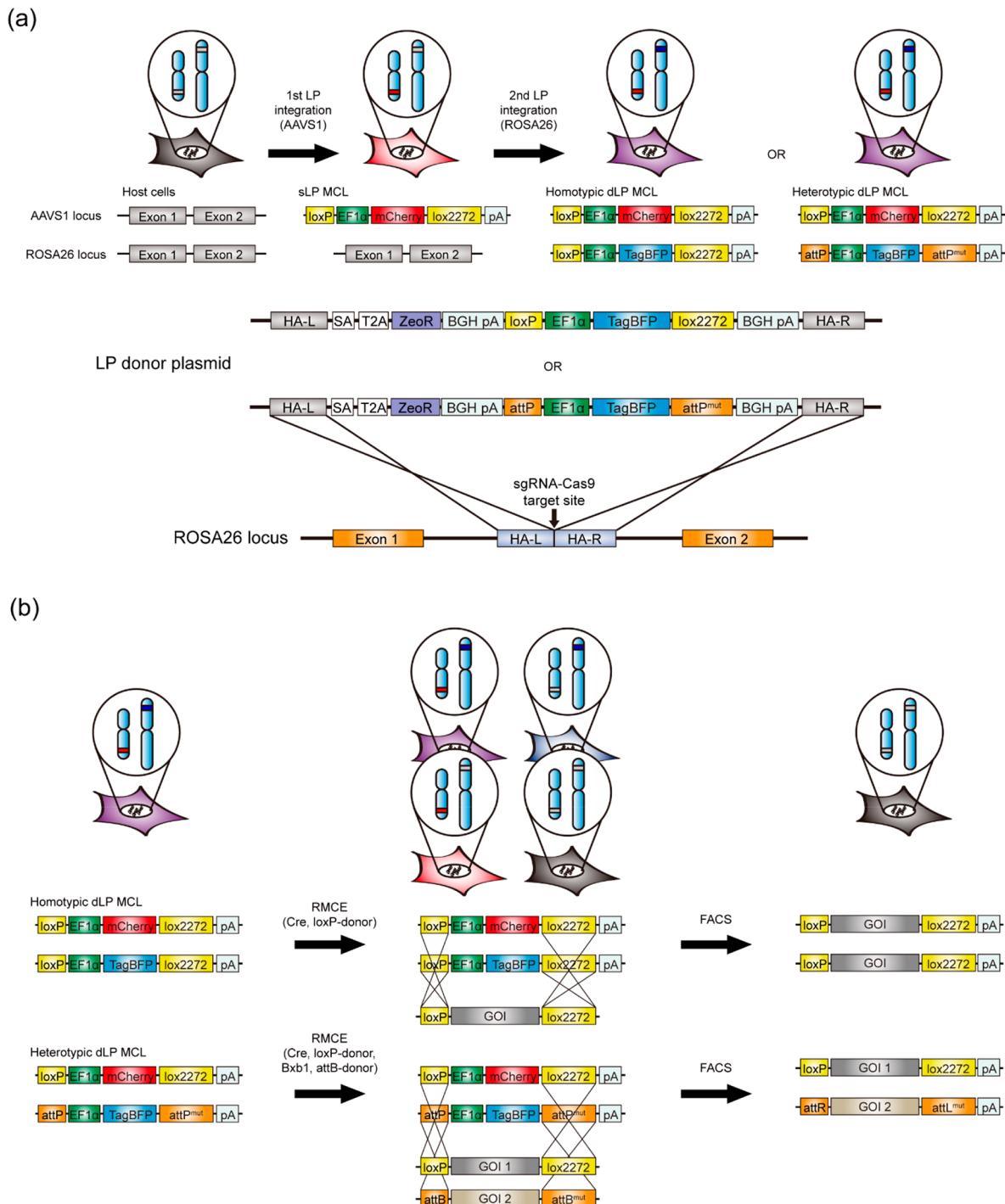
enables insertion of an exogenous gene cassette into a pretagged locus by replacing a target cassette, referred to as a landing pad, without any residual prokaryotic plasmid sequence.<sup>9</sup> Recombinase-mediated targeted integration offers reproducible transgene expression in reusable and predefined loci, thereby streamlining the timeline of cell line development.<sup>1</sup> Additionally, this strategy can be refined to guarantee the selection of producer cells in a drug-free manner without random integration of GOIs.<sup>2,10</sup>

A platform using RMCE coupled with CRISPR/Cas9 integrates a landing pad at a predefined locus in a genome and then exchanges the landing pad with a GOI using

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**Figure 1.** Generation of recombinant cell pools using an identical or orthogonal RMCE system. (a) A schematic diagram for two types of dual-landing pad (dLP) master cell lines (MCLs), homotypic and heterotypic, generated by CRISPR/Cas9-mediated integration of landing pad (LP) at the ROSA26 locus. A single-landing pad (sLP) MCL was transfected with sgRNA-Cas9 vector targeting the ROSA26 locus and LP donor plasmid. The LP donor plasmids consist of the zeocin selection element [splice-acceptor (SA), T2A, zeocin resistance gene, and BGH poly(A) signal (pA)] and LP sequences (EF1 $\alpha$  promoter and TagBFP gene, flanked by loxP and lox2272 or attP and attP $^{mut}$ ). (b) A schematic diagram for the generation of recombinant cell pools harboring dual copies of the transgenes by the RMCE. In homotypic dLP MCL, identical transgenes could be integrated at two safe harbors by transfection with Cre recombinase and loxP-donor. In heterotypic dLP MCL, different transgenes could be integrated at two safe harbors by transfection with Cre recombinase, loxP-donor, Bxb1 integrase, and attB-donor. To obtain recombinant cell pools harboring dual copies of the transgenes, mCherry-negative/TagBFP-negative cells were sorted using FACS.

recombinase, enabling the generation of isogenic clones.<sup>11</sup> Furthermore, this platform can be used for systematic evaluation of transgene expression under various conditions.<sup>12</sup> However, stable clones generated by targeted integration often

suffer from low expression levels of transgenes derived from a single copy of the GOI. To overcome this drawback, a hot spot of a genome, which ensured high and stable transgene expression, was screened and used for integration of a landing

pad.<sup>13–15</sup> A master cell line harboring a multi-landing pad was developed by CRISPR/Cas9-mediated integration using hot spots retrieved by random integration of a reporter gene, thus enabling predictable and reproducible expression of the transgene through recombinase-mediated integration of multiple copies of the gene.<sup>16,17</sup> The potential of this platform for efficient protein production and development of a synthetic gene network has been demonstrated by increasing the number of landing pads and GOI copies.<sup>16,17</sup>

Although the recombinase-mediated targeted integration has emerged as a promising tool for cell engineering, such as therapeutic protein production,<sup>18</sup> gene therapy,<sup>19</sup> construction of synthetic gene networks,<sup>20</sup> and library screening,<sup>21–23</sup> the low efficiency of recombination events without carrying out selection procedures often impedes its application. Recently, it has been reported that the efficiency of RMCE integration decreases as the size of the insert or number of landing pads increases.<sup>17</sup> Therefore, improvement of RMCE efficiency can facilitate the insertion of large gene cassettes at multiple sites, thereby streamlining the process of cell line development. Several studies have applied recombinase engineering approaches, such as tagging a nuclear localization signal (NLS) to recombinase<sup>24–27</sup> and developing recombinase fusion proteins that promote cellular uptake,<sup>28–31</sup> to improve recombination efficiency by enhancing the transport of recombinase. Delivery of DNA into the nucleus has also been performed to improve the efficiency of gene transfer. NLS peptide-conjugated plasmid DNA can be used to enhance the translocation of exogenous DNA.<sup>32–36</sup> DNA sequences, referred to as DNA nuclear-targeting sequences (DTSs), can be recognized by nuclear import proteins and exploited to promote DNA transport into the nucleus.<sup>37,38</sup> Enhancing nuclear transport of RMCE components, such as recombinase and donor plasmid, can support efficient recombination of gene cassette, thereby enabling the RMCE method to be a more amenable tool to be used in diverse fields related to cell engineering.

In this study, we generated two types of dual-landing pad (dLP) master cell lines (MCLs) harboring identical or orthogonal pairs of recombination sites, using two human genomic safe harbors (GSHs), AAVS1 and ROSA26 loci, as target sites in HEK293 cells. To investigate the effect of nuclear transport of recombinase, we constructed engineered Cre recombinase and Bxb1 integrase by adding three types of classical NLS sequences, SV40,<sup>39,40</sup> cMyc,<sup>41</sup> and nucleoplasmin (NP).<sup>42</sup> To improve the nuclear transport of donor plasmids, three types of DTSs, SV40,<sup>43</sup> glucocorticoid responsive element (GRE),<sup>44</sup> and nuclear factor- $\kappa$ B (NF- $\kappa$ B)<sup>45–47</sup> were added to the outside of gene expression cassettes. Next, combinatorial effects of the nuclear transport engineering components, NLS and DTS, were evaluated in both the dLP MCLs. Using this optimized RMCE system, we successfully generated recombinant cell lines by integrating identical transgenes at the AAVS1 and ROSA26 loci simultaneously by one-step transfection, thereby increasing the gene expression. Furthermore, this system enabled simultaneous exchange of different transgenes at two target sites, one site for product genes and another site for effector genes, resulting in improved productivity of recombinant proteins.

## RESULTS

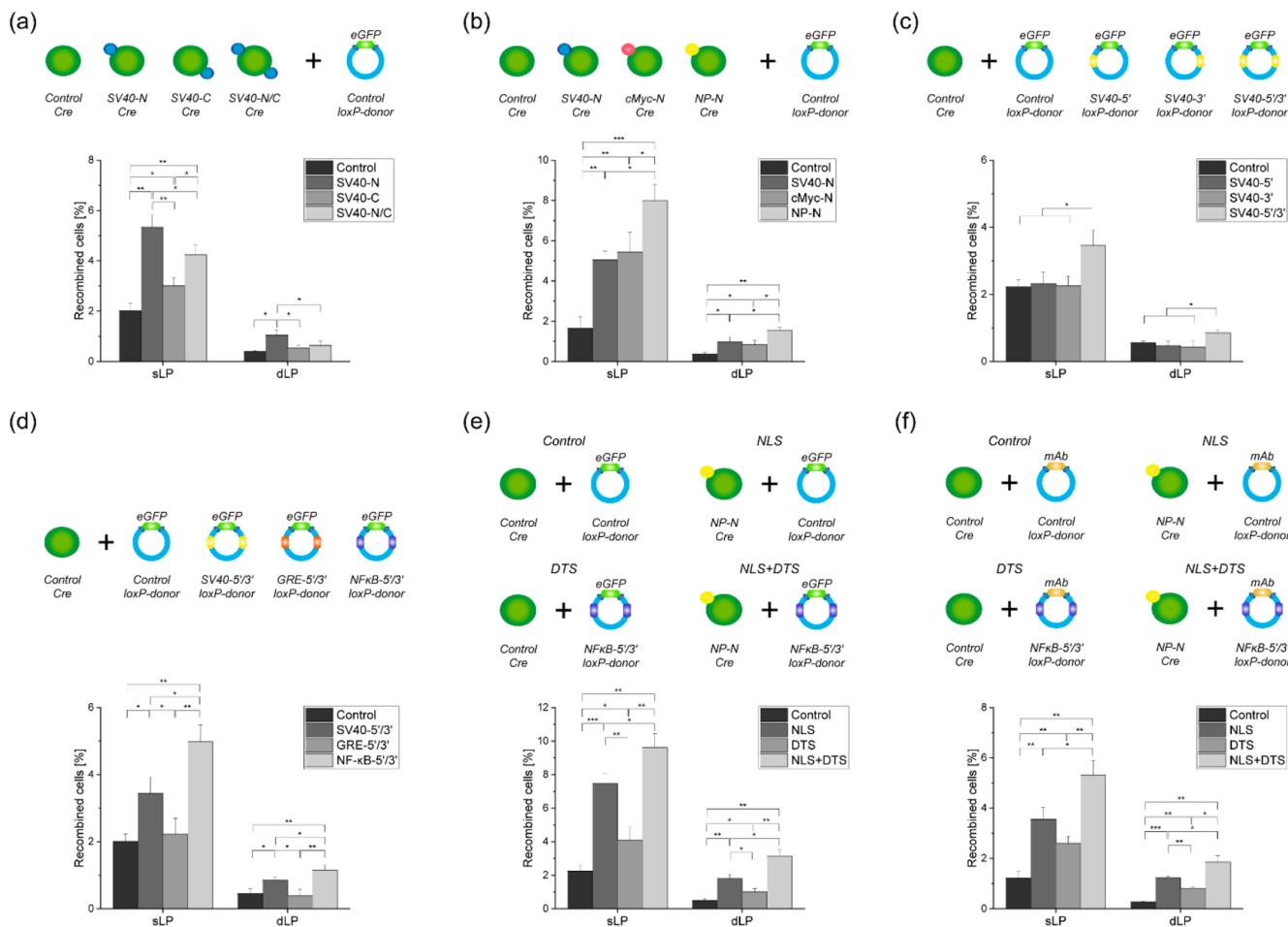
**Generation of MCLs Harboring dLP at GSHs.** For multigene expression at the AAVS1 and ROSA26 loci, stable

MCLs were generated by CRISPR/Cas9-mediated site-specific integration. A single-landing pad (sLP) MCL harboring a loxP-EF1 $\alpha$ -mCherry-lox2272 landing pad at the AAVS1 locus was constructed previously,<sup>48</sup> and a second landing pad, loxP-EF1 $\alpha$ -TagBFP-lox2272 or attP-EF1 $\alpha$ -TagBFP-attP<sup>mut</sup>, was integrated at the ROSA26 locus to generate homotypic or heterotypic dLP MCLs, respectively (Figure 1a). The sLP MCLs were transfected with LP donor plasmid and sgRNA-Cas9 plasmid targeting the ROSA26 locus. The transfected cells were subjected to zeocin selection for 2 weeks, and stable clones were isolated using limiting dilution of cell pools. Targeted integration of the landing pad at the ROSA26 locus was verified using 5'/3' junction PCR for each clone (Supporting Figure S1a). In order to select clones with two copies of the EF1 $\alpha$  promoter, quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the EF1 $\alpha$  promoter copy number, which was normalized to that of a control sample, sLP31, which harbors a single copy of the EF1 $\alpha$  promoter at the AAVS1 locus (Supporting Figure S1b).<sup>48</sup> Homotypic dLP MCL clone 19 and heterotypic dLP MCL clone 25 were chosen for further experiments.

Homotypic dLP MCL can be used to integrate two identical GOIs into each locus using RMCE. The MCL was transfected with two plasmids, loxP-donor and Cre recombinase, resulting in a cell pool containing four subpopulations: mCherry-positive/TagBFP-positive, mCherry-negative/TagBFP-positive, mCherry-positive/TagBFP-negative, and mCherry-negative/TagBFP-negative. To isolate the cell population expressing the GOI at both loci, the transfected pool was sorted for mCherry-negative/TagBFP-negative cells using FACS (Figure 1b). The use of heterotypic dLP MCL enabled the integration of two different GOIs by RMCE. The MCL was transfected with four plasmids, loxP-donor, Cre recombinase, attB-donor, and Bxb1 integrase, resulting in a cell pool of four subpopulations. The double-negative cells were sorted to isolate a cell population expressing two different GOIs (Figure 1b).

**Optimization of RMCE Condition for Stable Expression of GOIs.** To increase recombination events of the donor cassette into a landing pad, the ratio of transfection between donor and recombinase plasmids was optimized by titrating the amount of recombinase plasmid. The sLP MCL harboring a landing pad, loxP-EF1 $\alpha$ -mCherry-lox2272, at the AAVS1 locus was used to measure the RMCE efficiency of the Cre-lox system. When sLP MCL was transfected with the donor plasmid, EF1 $\alpha$ -eGFP flanked by loxP and lox2272, and Cre recombinase plasmid, mCherry-negative cells with eGFP protein expression were observed. RMCE efficiency was determined by calculating the percentage of mCherry-negative and eGFP-positive cells in the total number of cells. The Cre recombinase plasmid showed the best performance in terms of RMCE efficiency at a ratio of 64:1 (w/w) between the donor and Cre plasmids (Supporting Figure S2a).

Next, the heterotypic dLP MCL harboring a landing pad, attP-EF1 $\alpha$ -TagBFP-attP<sup>mut</sup>, at the ROSA26 locus was used to measure RMCE efficiency of the Bxb1-att system. When dLP MCL was transfected with the donor plasmid, EF1 $\alpha$ -eGFP flanked by attB and attB<sup>mut</sup>, and Bxb1 integrase plasmid, TagBFP-negative cells with eGFP protein expression were observed. RMCE efficiency was determined by calculating the percentage of TagBFP-negative and eGFP-positive cells in the total number of cells. The efficiency of RMCE was the highest



**Figure 2.** Improvement of RMCE efficiency in sLP and homotypic dLP MCLs. RMCE efficiency of eGFP donor in sLP and dLP MCLs with (a) different positions of SV40 NLS in Cre recombinase, (b) different types of N-terminal NLS in Cre recombinase, (c) different positions of SV40 DTS in loxP-donor, and (d) different types of 5'/3' DTS in loxP-donor. RMCE efficiency of (e) eGFP and (f) mAb donor in sLP and dLP MCLs with a combination of nucleoplasmin N-terminal NLS in Cre recombinase and NF- $\kappa$ B 5'/3' DTS in loxP-donor. Error bars represent standard deviations of three independent experiments. The data were analyzed using a one-way analysis of variance with Tukey's *post hoc* test, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

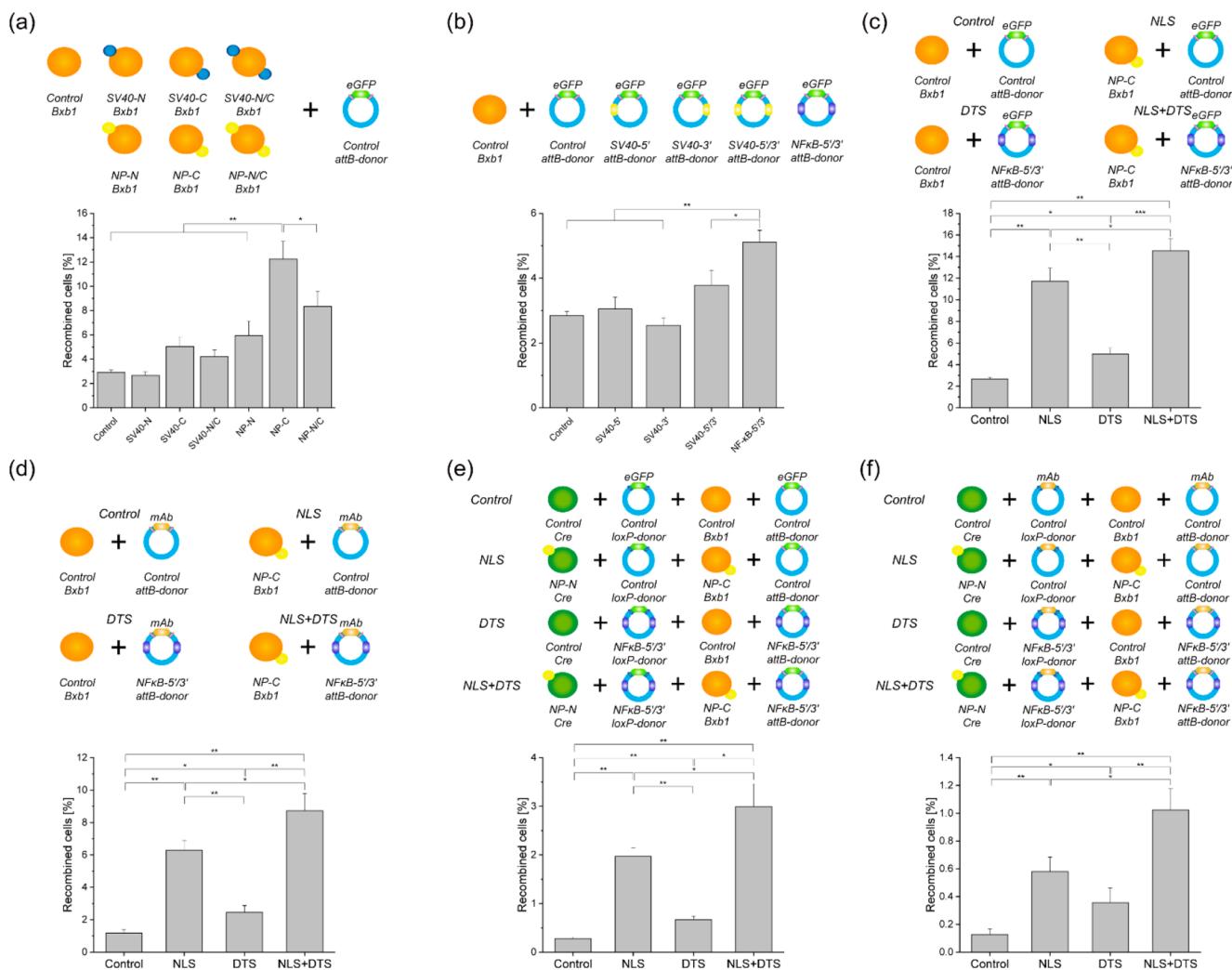
at a ratio of 4:1 (w/w) between the donor and Bxb1 plasmids (Supporting Figure S2b).

To assess the stability of recombinant cell pools, transfected pools were subcultured in 12-well plates for approximately three months. During the subculture, the percentage of recombined cells in the pools remained unchanged (Supporting Figure S3a). eGFP protein expression from the recombined donor cassette was also sustained at the AAVS1 and ROSA26 loci, at which the RMCE was mediated by Cre and Bxb1, respectively (Supporting Figure S3b).

**Accelerated Nuclear Transport of RMCE Components in Homotypic dLP MCL.** To facilitate multigene expression at GSHs, RMCE efficiency needs to be improved. The transport of RMCE components, such as recombinase protein and donor plasmid, can be accelerated by attaching NLS and DTS, respectively. The sLP and homotypic dLP MCLs were used for integration of the eGFP donor. To assess the effect of the position of NLS in Cre recombinase, Cre protein sequence was modified by adding an SV40 NLS to the N- or C-terminus, as well as to both the termini. Among them, the N-terminal NLS showed the highest efficiency in both sLP and homotypic dLP (5.3 and 1.1%, respectively), which was approximately 2.6 and 2.7 times higher than that of the control, respectively

(Figure 2a). Next, the effects of three classical NLSs, two monopartite NLS (SV40 and cMyc) and one bipartite NLS (NP), were compared in the sLP and dLP MCLs. Among the different types of NLS, NP N-terminal NLS showed the highest efficiency in both sLP and homotypic dLP (8.0 and 1.5%, respectively), which was approximately 4.8 and 4.3 times higher than that of the control, respectively (Figure 2b). Thus, improved nuclear transport of Cre recombinase by incorporation of NLS increased RMCE efficiency, and the N-terminal bipartite NLS showed the best performance, at both single and dual sites.

To assess the effect of the position of DTS in a donor plasmid, SV40 DTS was attached to the 5', 3', and both ends of the donor cassette. When DTS was added to both the ends, RMCE efficiency was increased in both sLP and homotypic dLP (3.5 and 0.9%, respectively), which was approximately 1.6 and 1.5 times higher than that of the control, respectively (Figure 2c). To determine the optimal DTS, the effects of three general DTSs, SV40, GRE, and NF- $\kappa$ B, were compared in sLP and dLP MCLs. As the length of each DTS was variable, 72, 27, and 10 bp for SV40, GRE, and NF- $\kappa$ B, respectively, one SV40 (72 bp), two GRE with a spacer (60 bp), and four NF- $\kappa$ B with five spacers (61 bp) were used to minimize the effect



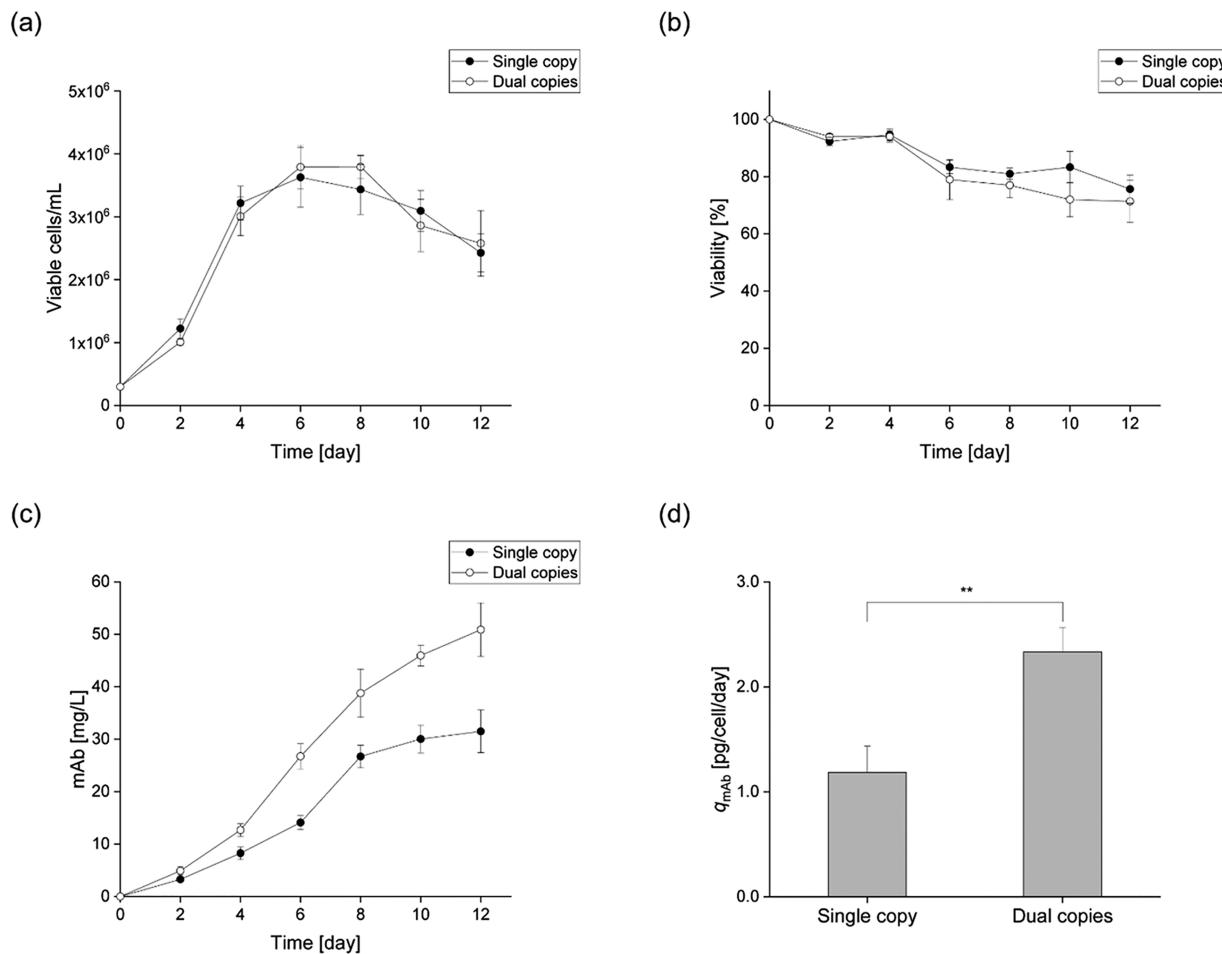
**Figure 3.** Improvement of RMCE efficiency in heterotypic dLP MCL. RMCE efficiency of eGFP donor in dLP MCL with different positions and types of (a) NLS in Bxb1 integrase and (b) DTS in attB-donor. RMCE efficiency of (c) eGFP and (d) mAb donor in dLP MCL with a combination of nucleoplasmin C-terminal NLS in Bxb1 integrase and NF- $\kappa$ B 5'/3' DTS in attB-donor. RMCE efficiency of (e) eGFP and (f) mAb donor in simultaneous integration using nucleoplasmin N-terminal NLS in Cre recombinase, NF- $\kappa$ B 5'/3' DTS in loxP-donor, nucleoplasmin C-terminal NLS in Bxb1 integrase, and NF- $\kappa$ B 5'/3' DTS in attB-donor. Error bars represent standard deviations of three independent experiments. The data were analyzed using a one-way analysis of variance with Tukey's *post hoc* test, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

of DNA length. NF- $\kappa$ B 5'/3' DTS was the most effective one in both sLP and homotypic dLP (5.0 and 1.2%, respectively), which was approximately 2.5 and 2.6 times higher than that of the control, respectively (Figure 2d). Thus, enhancement of DNA nuclear transport by incorporation of DTS into a donor plasmid can increase RMCE efficiency, depending on the position and type of DTS at both single and dual sites.

RMCE efficiency was measured in combination with NP N-terminal NLS and NF- $\kappa$ B 5'/3' DTS to examine the combinatorial effect of NLS and DTS. The highest efficiency was observed when both NLS and DTS were used in sLP and homotypic dLP (9.6 and 3.1%, respectively), which was approximately 4.3 and 6.3 times higher than that of the control, respectively (Figure 2e). To assess the effect of NLS and DTS on recombination of a large donor, a monoclonal antibody (mAb) expression cassette (3.9 kb *versus* eGFP donor of 1.9 kb), consisting of two promoters, two coding sequences, and a single poly(A), was used for RMCE in sLP and dLP MCLs. Similarly, RMCE efficiency showed the highest value when both NLS and DTS were used in sLP and homotypic dLP (5.3

and 1.9%, respectively), which was approximately 4.4 and 6.7 times higher than that of the control, respectively (Figure 2f). Representative FACS plots and gates are shown in Supporting Figure S4 and S5 for sLP and homotypic dLP MCLs, respectively.

**Accelerated Nuclear Transport of RMCE Components in Heterotypic dLP MCL.** Heterotypic dLP MCL, harboring two landing pads, one flanked by loxP and lox2272 and another flanked by attP and attP<sup>mut</sup> at the AAVS1 and ROSA26 loci, respectively, was used to assess the effect of NLS and DTS on the RMCE efficiency of the Cre-lox and Bxb1-att systems. NLS and DTS were added to Bxb1 integrase and the donor flanked by attB and attB<sup>mut</sup>, respectively. NP C-terminal NLS (Figure 3a) and NF- $\kappa$ B 5'/3' DTS (Figure 3b) showed the highest efficiency at the ROSA26 locus (12.2 and 5.1%, respectively), which was approximately 4.2 and 1.8 times higher than that of the control, respectively. To assess the combinatorial effect of NLS and DTS, NP C-terminal NLS and NF- $\kappa$ B 5'/3' DTS were used in eGFP and mAb donors. Using the eGFP donor, both NLS and DTS showed the highest



**Figure 4.** Profiles of (a) cell growth, (b) viability, and (c) mAb concentration during batch cultures of recombinant cell pools, expressing single or dual copies of mAb gene cassette. A single copy of mAb gene cassette (black circle) and dual copies of mAb gene cassette (white circle). (d)  $q_{mAb}$ . Error bars represent standard deviations of three independent experiments. The data were analyzed using an unpaired two-tailed *t*-test, \*\* $p < 0.01$ .

efficiency (14.5%), which was approximately 5.4 times higher than that of the control (Figure 3c). Similarly, the mAb donor showed the highest efficiency when both NLS and DTS were attached (8.7%), which was approximately 7.4 times higher than that of the control (Figure 3d).

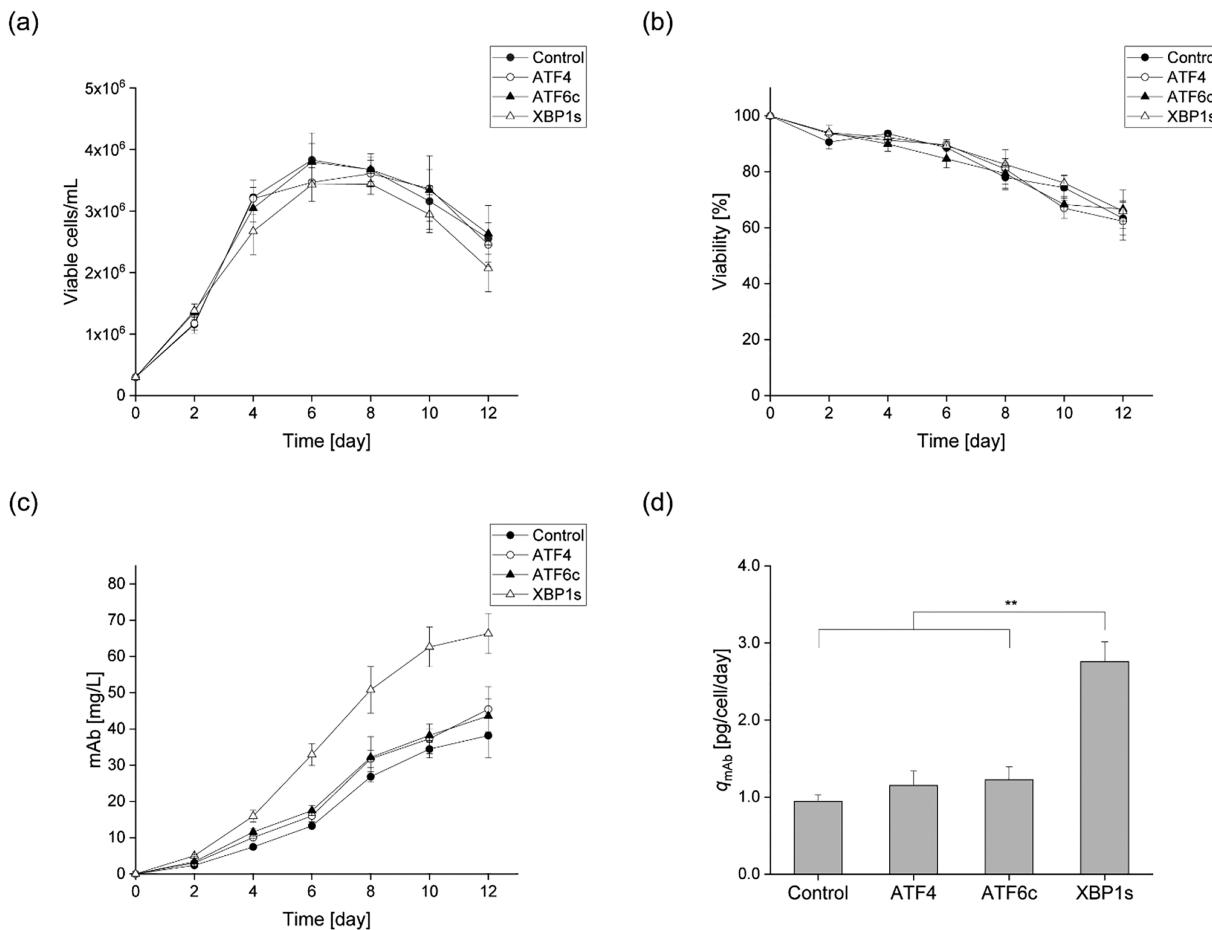
For simultaneous integration of two different donors, transfection of four plasmids, namely Cre, loxP-donor, Bxb1, and attB-donor, was performed in heterotypic dLP MCL. Incorporation of NP NLS in the Cre and Bxb1 increased RMCE efficiency 7.1 and 4.6 times compared to that of the control in eGFP and mAb donors, respectively (Figure 3e,f). Incorporation of NF- $\kappa$ B 5'/3' DTS in the donor plasmid also improved RMCE efficiency by 2.4 and 2.8 times compared to that of the control in eGFP and mAb donors, respectively (Figure 3e,f). When both NLS and DTS were used, RMCE efficiency showed the highest values, 10.8 and 8.1 times higher, compared to that of the control in eGFP and mAb donors, respectively (Figure 3e,f). Thus, this streamlined platform with accelerated nuclear transport enabled simultaneous integration of two donors, increasing the RMCE efficiency of Cre-lox and Bxb1-att systems up to practical selection levels of 3.0% and 1.0%, respectively. Representative FACS plots and gates are shown in Supporting Figure S6 and S7 for heterotypic dLP MCL.

#### Streamlined RMCE Platform Enabling Multigene Expression in Homotypic dLP MCL

To increase the

productivity of recombinant proteins such as mAb, homotypic dLP MCL can be used for the integration of GOIs at dual sites. Transfection with Cre recombinase and mAb donor plasmid was performed in sLP and homotypic dLP MCLs to generate recombinant cell pools producing mAbs from single and dual copies of genes, respectively. The recombinant cells were sorted by FACS using cells transfected with Cre recombinase as a gating control. As demonstrated earlier, NLS and DTS engineering increased the percentage of mCherry-negative and mCherry-negative/TagBFP-negative cells, which harbors mAb genes, in sLP and dLP MCLs, respectively, compared to that of the control (Supporting Figure S8). To assess mAb expression levels, recombinant cell pools harboring single or dual copies of the mAb genes were cultured in 12-well plates without selection pressure. Two cell pools showed similar growth profiles (Figure 4a,b), and the mAb titer was increased in the dual copies pool due to the higher specific mAb productivity ( $q_{mAb}$ ) of  $2.3 \pm 0.2$  pg/cell/day, which was 2.0 times higher than that of single copy pool (Figure 4c,d). Thus, the dual-RMCE system using homotypic pairs of recombination sites increased the expression level of recombinant proteins by doubling the copy number of transgenes in HEK cells.

**Streamlined RMCE Platform Enabling Multigene Expression in Heterotypic dLP MCL.** To facilitate simultaneous integration of different donors, heterotypic dLP MCL was transfected with the mAb donor cassette flanked by



**Figure 5.** Profiles of (a) cell growth, (b) viability, and (c) mAb concentration during batch cultures of recombinant cell pools, expressing a single copy of mAb gene cassette and UPR-related gene, ATF4, ATF6c, and XBP1s. Control (black circle), ATF4 (white circle), ATF6c (black triangle), and XBP1s (white triangle). (d)  $q_{mAb}$ . Error bars represent standard deviations of three independent experiments. The data were analyzed using a one-way analysis of variance with Tukey's *post hoc* test, \*\* $p < 0.01$ .

loxP and lox2272, Cre recombinase, effector genes flanked by attB and attB<sup>mut</sup>, and Bxb1 integrase. Unfolded protein response (UPR)-related transcription factors such as *ATF4*, cleaved *ATF6* (*ATF6c*), and *XBP1s*<sup>49</sup> were integrated by RMCE as effector genes, because such genes were evaluated in CHO cells with respect to the production of recombinant proteins.<sup>50–52</sup> The eGFP donor flanked by attB and attB<sup>mut</sup> was used to generate a control cell pool, and mCherry-negative/TagBFP-negative cells were sorted by FACS using cells transfected with Cre recombinase and Bxb1 integrase as a gating control (Supporting Figure S9). Protein expression of each target gene from the recombinant cell pools was confirmed by Western blotting (Supporting Figure S10). To assess productivity of the cell pools, cells were cultured in 12-well plates for batch culture, without selection pressure. Growth profiles of cell pools expressing *ATF4*, *ATF6c*, and *XBP1s* were similar to those of the control (Figure 5a,b). The mAb titer was increased in the *XBP1s*-expressing cell pool showing the highest  $q_{mAb}$  of  $2.8 \pm 0.2$  pg/cell/day, which was 2.9 times higher than that of the control (Figure 5c,d). Thus, recombinant cell pools expressing both therapeutic proteins and cell engineering targets could be generated using a single transfection in a dual-RMCE system using heterotypic pairs of recombination sites.

## DISCUSSION

Human cell lines are gaining attention as an alternative expression platform for therapeutic protein production because of human-like post-translational modifications, thereby ensuring proper activity of proteins and avoiding the risk of immunogenicity.<sup>53</sup> It has been reported that HEK293 producer cell pools achieved high titers of erythropoietin (EPO) with glycosylation profiles, which were similar to those of the endogenous protein,<sup>54</sup> and HEK293 cell lines have been used for the production of IgG-like bispecific antibodies by transient expression.<sup>55–58</sup> Furthermore, analysis of the human secretome has revealed that HEK293 cells can produce recombinant proteins difficult to produce in CHO cells.<sup>59</sup> Therefore, a streamlined platform for the generation of stable human cell lines is necessary for large-scale production of novel target proteins in a timely manner.

A traditional strategy for the generation of recombinant mammalian cell lines has relied on random integration of transgenes, which induces clonal variation and transgene rearrangement.<sup>60</sup> To overcome the drawbacks of random integration, targeted integration using CRISPR/Cas9 coupled with RMCE has been exploited because of stable and predictable expression of transgenes at a predefined site.<sup>11,12,17</sup> However, this system often suffers from low levels of recombination events as the size of a transgene increases,

particularly when the multiple copies of a gene needs to be exchanged with landing pads located at multiple sites. Therefore, enrichment of recombined cells using selection pressure, such as through the use of antibiotics, is required to obtain an adequate number of cells for analysis and characterization of recombinant cell lines. In this study, we describe a streamlined RMCE platform with improved recombination efficiency, which enables the generation of recombinant human cell lines expressing dual copies of transgenes at GSHs. This platform allows for the integration of dual copies of identical or different transgenes with a single transfection, using a single recombinase or two orthogonal recombinases, respectively.

Expression levels of recombinant proteins can be modulated by either increasing the copy number of a transgene, or by introducing an effector gene for host cell engineering.<sup>61</sup> Several RMCE-based strategies have attempted to integrate multiple transgene copies to improve therapeutic protein production. Using incompatible loxP sequences, multiple genes can be integrated at a single genomic locus, either sequentially<sup>62,63</sup> or simultaneously.<sup>64,65</sup> Furthermore, a multi-landing pad cell line enables the production of therapeutic proteins from multiple genes.<sup>16,17</sup> To increase the productivity of recombinant proteins, recombinant cells are often engineered by expressing effector genes. However, since the ectopic expression of effector genes relies on random integration, it shows inconsistent results due to clonal variation.<sup>66</sup> Transgenes used for cell engineering can also be integrated using RMCE, which is free from clonal variation. To find optimal conditions for the production of recombinant proteins, the expression of effector genes, such as transcription factors, needs to be modulated by gene dosage titration.<sup>67–69</sup> Therefore, the generation of cell lines expressing a single copy of a transgene enables an unbiased comparison of effector genes, and the expression level of effector genes can be controlled by increasing the copy number or changing the promoter of the effector genes.

Integration of two different transgenes can be a challenge in a dual-RMCE system, which requires simultaneous double exchange events. Targeting dual sites of the mammalian genome using recombinases can result in the integration of unwanted bacterial sequences,<sup>70,71</sup> or require sequential or simultaneous selection with two different antibiotics.<sup>72,73</sup> Two types of dLP MCLs, homotypic and heterotypic, were generated to express identical or different transgenes at GSHs. Homotypic dLP MCL can be used for the integration of two copies of a transgene by transfection with a single recombinase and donor. In CHO cells, it was demonstrated that the productivity of recombinant proteins was increased in correlation with the copy number of a transgene.<sup>16,17</sup> While homotypic dLP MCL enables an increase in transgene expression by doubling the copy number, heterotypic dLP MCL can be used for the integration of two different transgenes at designated sites. A cell pool expressing two different transgenes can be generated using a single transfection step with two types of recombinases and donors; however, the RMCE efficiency mediated by two different recombinases is very low because cotransfection efficiency of four large-sized plasmids is unreliable, and two pairs of different recombination sites need to be simultaneously exchanged with the corresponding donor cassette. Therefore, RMCE efficiency should be improved for the integration of a large gene cassette at multiple sites.

To increase the RMCE efficiency, we optimized the transfection conditions by titrating the amount of recombinase plasmid because recombinase induces cytotoxic effects in transfected cells.<sup>74</sup> The recombinase plasmid was serially diluted, and MCLs were transfected with a fixed amount of donor plasmid and different concentrations of the recombinase plasmid. A difference in RMCE efficiency was observed in both Cre recombinase and Bxb1 integrase, demonstrating that recombinase activity should be controlled to improve recombination efficiency (Supporting Figure S2).

Under these optimized RMCE conditions, the effect of enhanced nuclear transport of recombinase was assessed by incorporating NLS in the recombinase sequence. Because the position of NLS can affect the function of proteins,<sup>24,75</sup> we examined the RMCE efficiency in sLP and dLP MCLs, using Cre recombinase containing SV40 NLS at N- or C-terminus, as well as both the termini. It has been reported that the activity of PhiC31 integrase was affected by the position of NLS, because of its N-terminal catalytic domain.<sup>24</sup> Therefore, it can be inferred that N-terminal NLS supports the improved RMCE efficiency of Cre recombinase, which has a C-terminal catalytic domain.<sup>76</sup> When three classical NLSs were compared, the bipartite NP NLS showed the highest efficiency, similar to that shown in previous studies.<sup>77,78</sup> In Bxb1 integrase, the RMCE efficiency was increased by NLS attachment, and C-terminal NLS showed the highest efficiency in both SV40 and NP NLS, because of the N-terminal catalytic domain present in Bxb1 integrase.<sup>79</sup> Similar to Cre recombinase, the bipartite NLS was superior to the monopartite NLS with respect to recombination efficiency.

In addition to the nuclear transport of recombinases, nuclear transport of donor plasmids can be a bottleneck in RMCE, because transfected exogenous DNA needs to be imported into the nucleus through the nuclear pore complex. It has been reported that microinjection of plasmid into the nucleus showed a higher level of transgene expression than that of cytoplasmic injection in both nondividing and dividing cells.<sup>80</sup> DTS included in the plasmid sequence can be detected by several transcription factors present in the cytoplasm and form a complex with these proteins, thereby being imported into the nucleus.<sup>38</sup> We inserted DTS outside of the recombination sites in a donor cassette in order to remove this sequence after the RMCE process. Improvement in RMCE efficiency was observed only when both the 5' and 3' ends of the donor cassette contain the SV40 DTS in the RMCE mediated by Cre recombinase and Bxb1 integrase. Among the three types of general DTS, which are functional in all cell types,<sup>81</sup> NF- $\kappa$ B DTS showed the highest efficiency compared to the others in both Cre- and Bxb1-mediated RMCE processes.

To assess the effects of engineering in CHO cells, two sLP cell lines, which harbor a loxP-EF1 $\alpha$ -mCherry-lox2272 or attP-EF1 $\alpha$ -mCherry-attP<sup>mut</sup> landing pad, were used for RMCE. For NLS, NP N-terminal NLS in Cre recombinase and NP C-terminal NLS in Bxb1 integrase showed the highest RMCE efficiency, as demonstrated in HEK293 cells (Supporting Figure S11a,c). However, in the case of DTS, SV40 5'/3' DTS showed the highest RMCE efficiency for both sLP MCLs (Supporting Figure S11b,d). The variability in the abundance of transcription factors across different host cells may affect RMCE efficiency, because DTS needs to be recognized by transcription factors to transport the DNA into the nucleus. Similar to HEK293 cells, the highest RMCE efficiency was

observed when both NLS and DTS were used in CHO cells (Supporting Figure S11e,f).

The combination of NLS and DTS increased the RMCE efficiency; however, it was not an additive effect. Based on optimized conditions for RMCE, recombinant HEK293 cells that produced mAbs were generated by single transfection of recombinases and donor plasmids. In homotypic dLP MCL, two copies of mAb genes were integrated using Cre-mediated RMCE, and the productivity of mAb was increased 2-fold compared to that of the control. Furthermore, heterotypic dLP MCL was used to generate recombinant HEK cells harboring a single copy of mAb genes and effector genes related to the UPR pathway by Cre- and Bxb1-mediated RMCE. Therefore, this RMCE platform can be a useful tool for integrating identical or different donor cassettes at designated sites, enabling the modulation of transgene expression or excavation of effector genes for efficient production of specific target proteins, respectively.

In conclusion, the streamlined RMCE platform described in this study enabled multigene expression at GSHs by improving recombination efficiency of donor gene cassettes. Using homotypic and heterotypic dLP MCLs, rapid generation of stable recombinant cell pools expressing identical or different gene cassettes could be achieved by simultaneous integration without the need for antibiotic selection. This platform allows for modulation of transgene expression by increasing the copy number of gene cassettes, and the production of recombinant proteins can be enhanced by ectopic expression of effector genes. This human cell-based platform provides a favorable environment for difficult-to-produce proteins, which require proper modifications and processing.<sup>59</sup> In addition, the engineered RMCE system with improved recombination efficiency can be used in genome-wide CRISPR/Cas9 screening, enabling the construction of large-scale libraries with reduced cost.<sup>23</sup> This platform can be further improved by adjusting the strength of promoters, such as inducible and synthetic promoters,<sup>82,83</sup> to optimize transgene expression for the efficient production of recombinant proteins. Finally, it offers unique advantages as a universal platform for human cell engineering as well as cell line development by generating recombinant cell lines that stably express multigene cassettes at GSHs.

## METHODS

**Plasmid Construction.** All the plasmids used in this study are listed in Supporting Table S1 and were constructed by the uracil-specific excision reagent (USER) cloning method, as previously described.<sup>5</sup> All the primers used for USER cloning are listed in Supporting Table S2. For USER amplicons, PCR was performed using Phusion U Hot Start DNA polymerase (Thermo Fisher Scientific, Waltham, MA) with uracil-containing primers (IDT, Coralville, IA), according to the manufacturer's instructions. PCR fragments were purified using ExpiN Combo GP (GeneAll Biotechnology, Seoul, South Korea), and assembled using the USER enzyme (New England Biolabs, Ipswich, MA), followed by transformation using HIT Competent Cells DH5 $\alpha$  (RBC, Taipei, Taiwan), according to the manufacturer's instructions. The LP donor plasmids for homotypic or heterotypic dual-landing pad MCLs included 5' and 3' homology arms targeting the ROSA26 locus, zeocin resistance gene, loxP or attP, EF1 $\alpha$  promoter, TagBFP-coding sequence, lox2272 or attP<sup>mut</sup>, and BGH poly(A). The zeocin resistance gene was amplified from the

pcDNA3.1/Zeo(+) vector, and TagBFP-coding sequence was amplified from a plasmid as described previously.<sup>17</sup> The backbone sequence for the LP donor was amplified from pAAVS1-TLR targeting vector (Addgene plasmid # 64215, a gift from Ralf Kühn).<sup>84</sup> RMCE donor plasmids for the integration of eGFP included loxP or attB, EF1 $\alpha$  promoter, eGFP coding sequence, and lox2272 or attB<sup>mut</sup>. The EF1 $\alpha$  promoter and eGFP sequences were amplified from an RMCE eGFP donor plasmid.<sup>12</sup> The backbone sequence for an RMCE donor was amplified from RMCE EPO donor plasmid.<sup>12</sup> RMCE donor plasmids for the integration of mAb included loxP or attB, CMV promoter, LC coding sequence, BGH poly(A), CMV promoter, HC coding sequence, and lox2272 or attB<sup>mut</sup>. RMCE donor plasmids for the integration of UPR-related genes (ATF4, ATF6c, and XBP1s) included attB, EF1 $\alpha$  promoter, each target coding sequence, and attB<sup>mut</sup>. The ATF4, ATF6c, and XBP1s sequences were synthesized as gBlocks Gene Fragments (IDT). Cre recombinase and Bxb1 integrase coding sequences were derived from the PSF-CMV-CRE vector (Sigma-Aldrich, St. Louis, MO) and INCTbiosyn-pUB-HspINTBxb1 vector (Addgene plasmid # 127519, a gift from Martin Bonamino),<sup>85</sup> respectively. The CMV promoter, SV40 poly(A), and backbone sequence for the recombinase plasmid were amplified from PSF-CMV-CRE vector (Sigma-Aldrich). NLS sequences (SV40, cMyc, and nucleoplasmin) were included in USER primer, so as to be attached to the N- or C-terminus, as well as both the termini of recombinase. DTS (SV40, GRE, and NF- $\kappa$ B) were included in the USER primer to be attached to the 5', 3', and both ends of a donor cassette. All the plasmids were verified by sequencing and purified using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) and NucleoBond Xtra Midi EF Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Sequence information on the GOI, NLS, and DTS are listed in Supporting Table S3.

**Cell Cultivation.** HEK293E cells (ATCC number: CRL-10852) and sLP31 MCL were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin-streptomycin (Thermo Fisher Scientific), nonessential amino acid solution (Sigma-Aldrich), and GlutaMAX supplement (Thermo Fisher Scientific). Cells were grown in T25 flasks (Thermo Fisher Scientific), incubated at 37 °C with 5% CO<sub>2</sub> and passaged every 3 days. Viable cell concentration and viability were estimated using a CountessII FL automated cell counter (Invitrogen, Carlsbad, CA) by the trypan blue dye exclusion method. Cell lines were tested for mycoplasma contamination using PCR and were found to be negatively contaminated.

**Generation of Dual-Landing Pad Master Cell Lines.** The sLP31 MCL was seeded at  $0.5 \times 10^6$  cells/mL in T25 flasks containing 5 mL culture medium. After 24 h, cells were transfected with sgRNA-Cas9 vector targeting the ROSA26 locus and LP donor plasmids (loxP or attP), at a ratio of 1:1 (w/w), using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cell pools were established by selection with 200  $\mu$ g/mL zeocin (Invitrogen) for 2 weeks. Next, stable cell lines were generated using limiting dilution method, seeding the cell pools at 0.3 cells/well into 96-well plates.

**5'/3' Junction PCR.** Genomic DNA was extracted from stable cell lines in 48-well plates using QuickExtract DNA extraction solution (Epicenter, Illumina, Madison, WI),

according to the manufacturer's instructions. PCR was performed using PrimeSTAR HS Premix (Takara Bio, Shiga, Japan) by touchdown PCR (98 °C for 3 min; 10 × cycles: 98 °C for 10 s, 68–58 °C (−1 °C/cycle) for 30 s, 72 °C for 2 min; 30 × cycles: 98 °C for 10 s, 58 °C for 30 s, 72 °C for 2 min; 72 °C for 10 min). The primers used in junction PCR are listed in *Supporting Table S2*.

**Measurement of Gene Copy Number by qRT-PCR.** Genomic DNA was purified from  $3.0 \times 10^6$  cells in 6-well plates using Exgene Blood SV (GeneAll Biotechnology). Analysis of gene copy number was performed using the CFX96 Real-Time System (Bio-Rad, Hercules, CA) and iQ SYBR Green Supermix (Bio-Rad), as previously described.<sup>86</sup> Relative copy number of the EF1 $\alpha$  promoter was calculated using the  $\Delta\Delta CT$  method, normalized to human ACTB gene. The primers used in qRT-PCR are listed in *Supporting Table S2*.

**Generation of Recombinant Cell Pool.** MCLs were seeded at  $0.5 \times 10^6$  cells/mL in 12-well plates containing 1 mL culture medium. After 24 h, the cells were transfected with donor and recombinase plasmids at a ratio of 64:1 (w/w) for Cre and 4:1 (w/w) for Bxb1, using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 8 days, recombinant cell pools were sorted by MoFlo Astrios EQ (Beckman Coulter, Brea, CA) to isolate mCherry-(488 nm blue laser with 620/29 filter), TagBFP- (405 nm violet laser with 448/59 filter), and double-negative populations.

**Titration of Recombinase Concentration.** Cre recombinase or Bxb1 integrase plasmids were serially diluted 2-fold, from 250 to 3.91 ng or 1000 to 15.6 ng, respectively. Recombinase prepared at seven different concentrations and a fixed amount of donor plasmid (1  $\mu$ g) were used for transfection using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. RMCE efficiency at each condition was measured using flow cytometry.

**Stability of Recombinant Cell Pool.** Cell pools harboring the eGFP gene at the AAVS1 or ROSA26 loci by RMCE were passaged every 3 days for approximately three months in 12-well plates containing 1 mL of culture medium. At every passage, the initial viable cell concentration was  $0.2 \times 10^6$  cells/mL. RMCE efficiency and the expression levels of eGFP protein were measured by flow cytometry at passage 0, 10, 20, and 30. The expression level of eGFP protein was normalized to that of the AAVS1 sample at passage 0. To avoid measurement error, control cell samples expressing eGFP protein were analyzed along with test samples at each passage.

**Flow Cytometry.** For analysis of RMCE efficiency, transfected cells in 12-well plates were prepared in phosphate-buffered saline, as previously described.<sup>87</sup> Expression levels of mCherry (488 nm blue laser with 610/20 filter), TagBFP (405 nm violet laser with 450/50 filter), and eGFP (488 nm blue laser with 530/30 filter) proteins in 100 000 cells were analyzed using FACS LSRFortessa (BD Biosciences, San Jose, CA). For an eGFP donor, RMCE efficiency was determined by calculating the percentage of mCherry-negative and eGFP-positive cells (AAVS1 locus), TagBFP-negative and eGFP-positive cells (ROSA26 locus), and double-negative and eGFP-positive cells (AAVS1 and ROSA26 loci) in the total number of cells. For the mAb donor, RMCE efficiency was determined by calculating the percentage of mCherry-negative cells (AAVS1 locus), TagBFP-negative cells (ROSA26 locus),

and double-negative cells (AAVS1 and ROSA26 loci) in the total number of cells.

**Batch Cultivation.** Cells were seeded at  $0.3 \times 10^6$  cells/mL in 12-well plates containing 1 mL culture medium and grown at 37 °C with 5% CO<sub>2</sub>. Viable cell concentrations and viability were measured in a single well every 2 days. For the measurement of titers, culture supernatants were harvested every 2 days and stored at −70 °C for further analyses.

**Measurement of mAb Concentration.** The titer of mAbs in culture supernatant was determined using an enzyme linked immunosorbent assay, as previously described.<sup>88</sup>  $q_{mAb}$  was calculated using the values of integral viable cell concentration and the titer in exponential phase (from day 2 to 6), as previously described.<sup>89</sup>

**Western Blot Analysis.** Protein expression of UPR-related genes (ATF4, ATF6c, and XBP1s) was measured by Western blot analysis, as previously described.<sup>88</sup> Total proteins were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, and 12  $\mu$ g of each protein extract was loaded per well. Proteins were transferred to polyvinylidene difluoride membranes using the iBlot2 Dry Blotting System (Thermo Fisher Scientific), and blocking was performed using 5% Difco Skim Milk (BD Biosciences). The antibodies used for immunoblotting were anti-ATF4 rabbit antibody (Cell Signaling Technology, Danvers, MA), anti-ATF6 mouse antibody (Novus, St. Charles, MO), anti-XBP1s rabbit antibody (Cell Signaling Technology), anti- $\beta$ -actin mouse antibody (Sigma-Aldrich), antimouse IgG, HRP-conjugated antibody (KOMABIOTECH, Seoul, South Korea), and antirabbit IgG, HRP-conjugated antibody (Cell Signaling Technology). The bands were detected using ECL Western Blotting Detection Reagents (GE Healthcare, Chicago, IL) and EZ-Western Lumi Femto (DoGenBio, Seoul, South Korea), and the images thus obtained were analyzed using ChemiDoc (Bio-Rad).

**Statistical Analysis.** Statistical significance was calculated using one-way analysis of variance with Tukey's *post hoc* test or unpaired two-tailed *t*-test;  $p < 0.05$  was considered significant.

## ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.1c00113>.

Generation of dual-landing pad (dLP) master cell lines (MCLs) using the CRISPR/Cas9-mediated integration; titration of recombinase plasmid concentration; stability of recombinant cell pools; representative FACS plots and gates of RMCE in the sLP MCL; representative FACS plots and gates of RMCE in the homotypic dLP MCL; representative FACS plots and gates of RMCE in the heterotypic dLP MCL; representative FACS plots and gates of simultaneous RMCE in the heterotypic dLP MCL; generation of recombinant cell pools producing mAbs from single or dual copies; simultaneous cassette exchange of mAb and effector genes related to the UPR pathway; protein expression of effector genes related to the UPR pathway, ATF4, ATF6c, and XBP1s, in recombinant cell pools; improvement of RMCE efficiency in CHO cells; plasmid information; primer sequences; sequence information (PDF)

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S.S., J.S.L., and G.M.L. designed the experiments. S.S. and S.H.K. conducted the experiments. S.S., J.S.L., and G.M.L. wrote the manuscript. All authors read and approved the manuscript.

### Notes

The authors declare no competing financial interest.

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