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Novel Tag-and-Exchange (RMCE) Strategies Generate Master Cell Clones with Predictable and Stable Transgene Expression Properties

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Site-specific recombinases have revolutionized the systematic generation of transgenic cell lines and embryonic stem cells/animals and will ultimately also reveal their potential in the genetic modification of induced pluripotent stem cells. Introduced in 1994, our Flp recombinase-mediated cassette exchange strategy permits the exchange of a target cassette for a cassette with the gene of interest, introduced as a part of an exchange vector. The process is "clean" in the sense that it does not co-introduce prokaryotic vector parts; neither does it leave behind a selection marker. Stringent selection principles provide master cell lines permitting subsequent recombinase-mediated cassette exchange cycles in the absence of a drug selection and with a considerable efficiency (~10%). Exemplified by Chinese hamster ovary cells, the strategy proves to be successful even for cell lines with an unstable genotype.

Keywords: site-specific recombination; RMCE; targeted integration; tag and exchange; gene expression

Introduction

During recent years, the market for biopharmaceuticals has widely expanded and is expected to reach US$70 billion by 2010.[1] Major products are recombinant proteins, produced in cultivated mammalian cell lines, among which the Chinese hamster ovary (CHO) line is the most prominent one.[2,3] Traditionally, a transgene under the control of potent transcription regulatory elements is randomly integrated into the host chromosomes together with a selectable marker gene. A drug-selection procedure in media containing a cytotoxic antibiotic or devoid of an essential metabolic enzyme is then applied for the generation of stable transgenic cell lines.[4] For such a process, the expression level of transfected cells is variable and unstable, mostly due to the unpredictable influence of random integration sites (position effect). A further complication arises from the presence of multiple integrated copies, which tend to cause recombination, chromosomal aberration[5] and/or repeat-induced silencing.[6] The fact that high producers account for a minor

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Abbreviations used: CHO, Chinese hamster ovary; GOI, gene of interest; FRT, Flp recognition target; RMCE, recombinase-mediated cassette exchange; FACS, fluorescence-activated cell sorting; S/MAR, scaffold/matrix attachment region; HDACi, histone deacetylase inhibitor; NaBu, sodium butyrate; VPA, valproic acid; ES, embryonic stem; HSV, herpes simplex virus; Ganc, ganciclovir; eGFP, enhanced green fluorescent protein; HR, homologous recombination; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PI, propidium iodide.

proportion of the transfected cells calls for immediate screening since low producers tend to overgrow highly expressed cells, for which metabolic resources are diverted to the expression of the gene of interest (GOI).[7] Finally, even if a transcriptional hotspot is hit by incidence, a random screening process cannot guarantee the recurrence of such an event.

Site-specific recombinases mediate the targeted integration of a single gene copy into a pre-identified hotspot and have enabled a platform for the reproducible establishment of high producers. As an example, according to the 'Flp-in' principle,[8,9] a transgene can be inserted into pre-tagged locus *via* a reciprocal crossover between identical Flp recognition target (FRT) sites, one on the target and the second one on the targeting vector.[10,11] In the persistent presence of Flp recombinase, this event will be immediately reversed in an entropy-driven excision reaction unless a sub-fraction can be captured by a stringent selection principle.[12] Even in this case, transcription of the GOI may be affected by the co-expressed selection marker, which *per se* is subject to host defense directed against the prokaryotic vector parts.[13] Other site-specific recombinase systems suffer from the same category of problems.[14,15] Only with the advent of more advanced recombinase-mediated cassette exchange (RMCE) techniques has the direct replacement of a selection marker for the GOI become possible. The RMCE principle involves a double reciprocal crossover between identical sets of sites consisting of two heterospecific FRTs that flank both the genomic target and the targeting cassette.[16]

These properties have motivated the application of RMCE for the generation of various production cell lines.[17–20] Although such a "tag-and-exchange" strategy has properties preventing host-defense mechanisms, major efforts had still to be undertaken to overcome consequences that arise from the unstable karyotype of the CHO cell line, that is, the identification of hotspots that are amenable to the RMCE principle. These loci enable high and persistent transcription levels and remain accessible to both the expression and the recombination machineries.

Ideally, the targeted integration events can be recovered in the absence of any drug selection to avoid cellular stress-related phenomena[21] and to overcome the documented repressive effects of selection markers adjacent to the GOI.[22,23] In order to reduce the risk of antibiotic contaminants in industrial fermentation processes, cell sorting [e.g., fluorescence-activated cell sorting (FACS)] techniques have emerged as an attractive alternative, as they prevent unwanted drug-resistance phenomena. Under circumstances where the RMCE approach enables the integration of a single copy of the GOI into the tagged site, a high degree of homogeneity and stability in gene expression can be anticipated.[24] Although, by nature, the screening for suitable target sites mediating long-term high expression is time-intensive, it has to be performed only once as the resulting master clones lend themselves to repeated usage.

During the replication cycle, an inserted transgene becomes wrapped by histones to adopt its final chromatin structure. The nature of the respective site determines the performance of the transcription control elements and whether or not position-effect variegation occurs. In this context, the possibility to improve the site's characteristics by the addition of genomic insulators and/or bordering elements has gained particular attention. Flanking the GOI by scaffold/matrix attachment regions (S/MARs) has been reported to protect the expression cassette from heterochromatization[25–28] and to make it susceptible to the augmenting effects of histone deacetylase inhibitors (HDACis) such as sodium butyrate (NaBu), valproic acid (VPA) and trichostatin A.[28–30]

In the present work, clones with highly expressed loci are evaluated using a fluorescent marker, *d2egfp*, before the clone mixture is subjected to a recombination competence test consisting of two consecutive rounds of RMCE. In RMCE 1, the *gfp* cassette is exchanged for a *hygtk* (hygromycin/thymidine kinase fusion gene) cassette. Cells that lose their fluorescence as anticipated are collected by FACS counterselection. For the majority of nonfluorescent cells, fluorescence is restored in RMCE 2, which reintroduces a related but different *gfp* cassette. The resulting population gives rise to master clones with persistently exchangeable loci, well suited for the ultimate introduction of the GOI.

The success of this procedure largely depends on a novel "promoter trap" strategy, which efficiently circumvents disturbances from nonspecific integration events. Since the exchange vectors lack a functional upstream control region, their expression depends on the promoter residing next to the target cassette within the recipient clone. As a consequence, production cells can be FACS selected in the absence of any drug. Clones arising from this population maintain their expression over extended periods of time, whether or not S/MAR flanks have been added to the parental cassette. Apparently, all sites arising from our selection procedure provide S/MAR-like features as their expression is augmented by the mentioned HDACi agents triggering histone hyperacetylation.

Results

Clones with unique retargetable genomic sites

RMCE techniques have been developed to guide transgenes into a predefined genomic locus with the desired expression properties. Recent work by Kim and Lee demonstrates the potential of this concept for CHO cells but leaves open the question how cell clones with a highly expressed, stable and retargetable site can be efficiently isolated.[17] The preselected integration sites should meet the following criteria to be of use for routine procedures:

1. They should be present in a stable genomic locus with high transcriptional potential.

2. They should be targetable in the absence of drug selection and with reasonable efficiency to reduce the number of clones that have to be screened.
3. They should accommodate a single copy of the GOI; no other integration events should occur elsewhere.

A system with these properties would overcome the instability and the associated variable expression caused by multiple genomically anchored copies.[31]

Pilot experiments

We and others have successfully applied RMCE for embryonic stem (ES) cell engineering in the past.[32–34] Based on this experience, we tried to verify the classical tag-and-exchange strategy[33] on CHO-K1 cells in a pilot experiment. The cells were tagged by a parental cassette containing an *hygtk* as a positive/negative (+/−) selection marker, flanked by a set of FRT sites. This set consists of an FRT^{wt} site ("F", containing the unchanged spacer sequence TCTAGAAA) and an FRT mutant FRT3 ("F3", spacer sequence TTCAAATA).[16] Rather than screening for single-copy hygromycin-resistant clones already at this point, the entire pool (designated "C1") that survived Hyg selection was subjected to RMCE. In the common case of multiple head-to-tail integrated copies, once applied, Flp recombinase would reduce a concatemer to the single-copy level *via* the excision pathway (crossovers between F × F or F3 × F3 sites).[35]

RMCE (in addition to excision) was then initiated by the co-transfection of the Flp expression construct together with a circular "exchange vector" for which the GOI is flanked by the same set of FRTs (Fig. 1a). At the transient stage, the highest expressers were sorted out by FACS (Fig. 1b) and subjected to ganciclovir (Ganc) "negative" selection.[37] As a pro-drug, Ganc excludes non-exchanged parental cells once it has been converted to a toxic, phosphorylated nucleotide analog by the herpes simplex virus (HSV) suicide thymidine kinase (*tk*) gene. The loss of *hygtk* by exchange for the *d2egfp* cassette generated fluorescence according to the RMCE principle, which, in turn, served as a traceable marker to evaluate, by FACS, the transcription level of targeted cells.[38] From the population of surviving cells with a high d2eGFP expression (termed "10/06-C1-S", in Fig. 1d), candidate clones can be isolated by FACS.

A genomic target might profit from the presence of boundary elements such as S/MARs[28] by which they are shielded, for instance, from the silencing effects of a heterochromatic environment. Therefore, we performed an analogous series of experiments (Fig. 1e–g) in which the parental cassette was flanked by two well-established S/MARs. Their presence apparently leads to largely improved exchange efficiency (see the higher contribution of strongly expressed cell in Fig. 1f as compared to Fig. 1c). Since other facts (covered in Discussion) may also have contributed to this difference, we screened the population recovered in Fig. 1d for cells that owe their fluorescence exclusively to an RMCE-mediated integration event at a single targetable site.

Even in this case, complications were anticipated from PCR analyses on the sorted d2eGFP-expressing pool (Fig. 1d):

- The first PCR (Fig. 2a) was performed to trace authentic exchange events within the enriched high expressers, 10/06-C1-S. The primer pair p2494/pGFP6 (indicated in Fig. 1a) was designated to trace the targeted integration of the exchange cassette (F3-P^{cmv}*d2egfp*-F), as this causes a unique 1. 0-kb segment in case of the RMCE-mediated integration (Fig. 2a).
- The second PCR (Fig. 2b) was performed to find out whether mere silencing of the *tk* gene in the parental cassette had led to the survival of some non-exchanged cells in the 10/06-C1-S population. This question was raised by the fact that, after RMCE induction and selection, the majority of cells (>90%) indicated spontaneous resistance to Ganc by their nonfluorescence. The primer pair p2494/p2471 (Fig. 1a) was designed to trace the parental cassette (F3-*hygtk*-F). Upon amplification, it gave rise to a 520-bp band for 10/06-C1-S cells as well as C1 cells (positive control).
- The third PCR (Fig. 2c) was performed to clarify whether random integration of the exchange plasmid also contributed to the d2GFP expression of 10/06-C1-S. Figure 1a indicates the relevant primer pair p1230/p2484, which gave rise to a 900-bp amplified fragment for the exchange vector (F3-P^{cmv}*d2egfp*-F; positive control). In fact, randomly integrated F3-P^{cmv}*2egfp*-F vectors were found for 10/06-C1-S (no signal was observed for parental cells, C1 as a negative control).

These results together reflect the complexity of the cell mixture arising after RMCE by Ganc counterselection. First of all, not only the RMCE-targeted *d2egfp* cassette but also randomly integrated *d2egfp* vectors contribute to the fluorescence of the cell mixture; the latter situation is rarely found in ES cells, which integrate linearized rather than circular DNA.[39] Secondly, the counterselection with Ganc is insufficient to eliminate all non-exchanged parental cells,[40] which may have developed spontaneous resistance to Ganc *via* epigenetic silencing of *tk* functions.[33]

Due to this complexity and to an unknown exchange rate, intense screening would have to follow in order to recover the desired events. In addition, the strategy does not include the means to select for a single-copy integration event, that is, a unique targetable site. Since close to nothing is known about the correlation between a high expression level and the RMCE competence of a given site,[41] a randomly integrated P^{cmv}*d2egfp* unit might occur among the high expressers. Based on

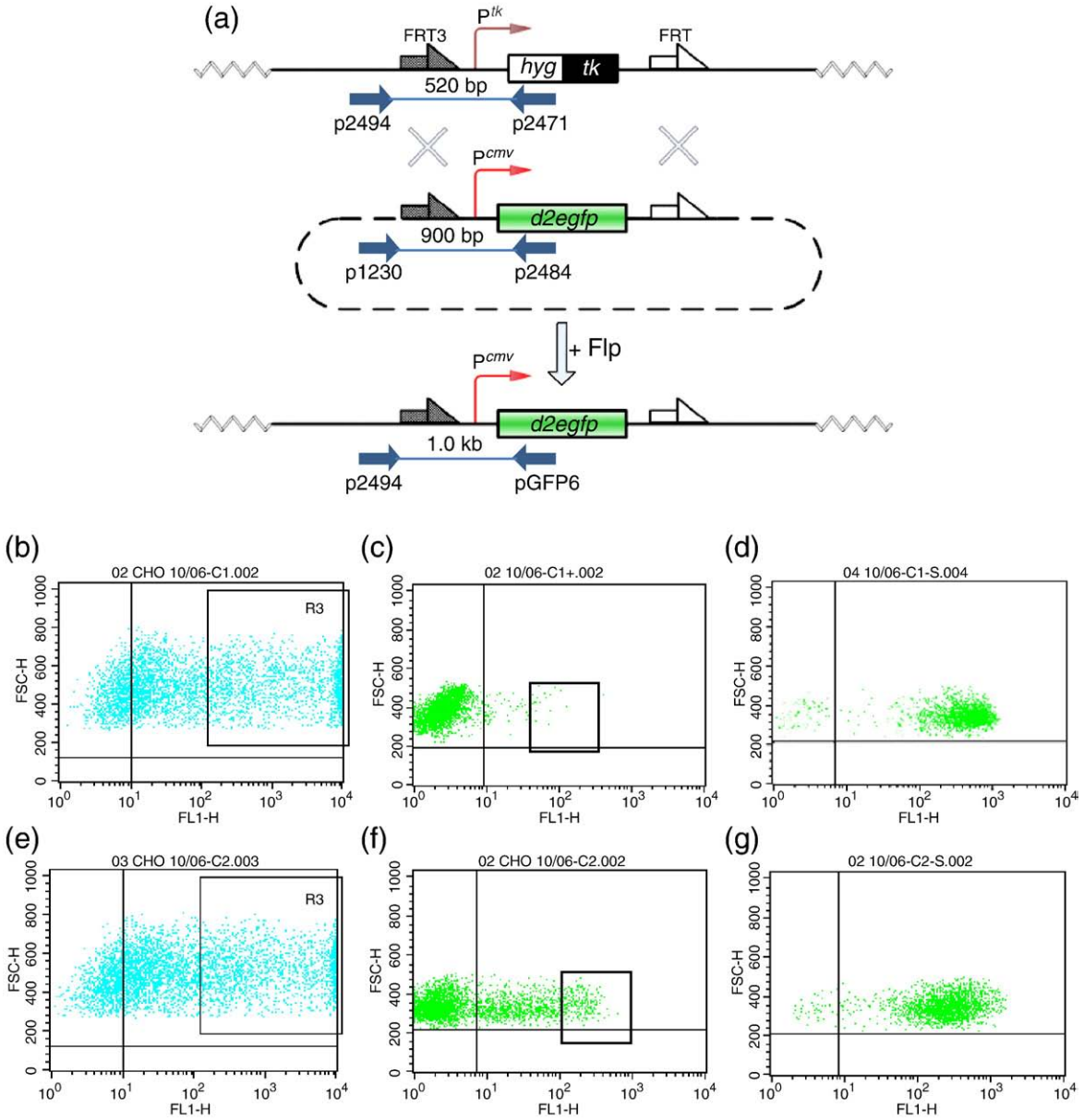

Fig. 1. Pilot experiments with the tag-and-exchange strategy. (a) Outline of the RMCE strategy: The parental vector contains a hygromycin and thymidine kinase (*hygtk*) fusion gene under control of the thymidine kinase (*tk*) promoter (P^{tk}). The parental cassette is flanked by a set of heterospecific FRT sites, the set consisting of the FRT mutant "FRT3" ("F3") and a wild-type FRT site, "F". The ScaI-linearized parental cassette was electroporated into CHO-K1 cells, and a pool of Hyg-resistant cells (designated C1) was selected. The circular exchange vector accommodates the exchange cassette [encoding a d2eGFP expression unit controlled by the cytomegalovirus (*cmv*) promoter (P^{cmv})], embedded by prokaryotic vector sequences (broken line). Authentic RMCE leads to the loss of the dashed vector part. The process is triggered by the co-transfection of an Flpe expression vector, into C1 cells. The *flpe* gene is part of a bicistronic *flpe-IRES-puromycin* (*flpe-pac*) cassette. (b–d) Enrichment processes of potentially targeted clones. (e–g) Corresponding data for a cassette in which the F3-*hygtk*-F cassette is flanked by two S/MAR elements (the 2. 2-kb element E upstream and the 1. 3-kb element W downstream[36]). (b and e) Transient expression phase: After the transfected cells were treated with puromycin (Puro) for 2 days, 20–30% of highly expressing cells were sorted by FACS and then subjected to Ganc selection. (c and f) Ganc-resistant cells were sorted once more to obtain 1% (c; population "10/06-C1") or 10% (f) highest-expressing cells. (d and g) Double-sorted cells showed stable fluorescent expression over the entire test period (typically 2 months). The population sorted out in (c) has been termed 10/06-C1-S.

these considerations, we refined our strategy by including the following selection steps:

- the tagged clonal pools are preselected for high expression before being tested for RMCE competence;
- interferences from random integration of the exchange cassette are eliminated by a straightforward promoter trap;
- preselection for unique targetable sites is applied in order to reduce the number of clones to be screened.

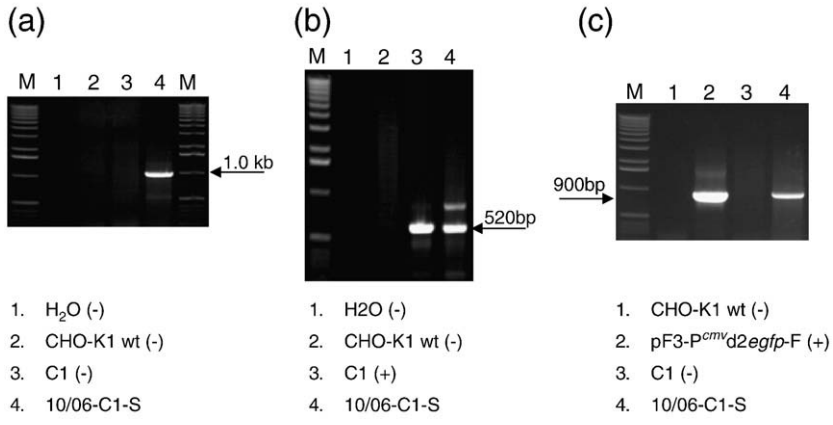

Fig. 2. PCR analyses on the potentially targeted cells from Fig. 1. (a) PCR to verify authentic exchange events in the enriched d2eGFP-expressing cells 10/06-C1-S with primer pair p2494/GFP6 as indicated in Fig. 1a (bottom part): PCR reactions were performed on H_2O, CHO-K1 genomic DNA and parental C1 cells as negative controls (−). (b) Examination of remaining (silenced) parental cassettes in C1 cells before [as a positive control (+)] and after exchange (10/06-C1-S); PCR reactions were performed with primer pair p2494/2471 as indicated in Fig. 1a (top). Negative controls (−) were based on CHO-K1 cells and on H_2O. (c) Verification of randomly integrated exchange cassettes by PCR with primer pair p1230/2484 as indicated in Fig. 1a (center). Negative controls (−) were based on PCRs on CHO-K1 genomic DNA and on untreated C1 cells. PCR on the exchange plasmid F3-P^{cmv}d2*egfp*-F served as a positive control (+). M: 1. 0-kb marker.

The refined strategy

In order to meet these criteria, the strategy is based upon two consecutive rounds of RMCE (Fig. 3). Other than in our pilot experiment, CHO-K1 cells are tagged by a cassette (P^{cmv}F3-*d2egfp*-F), which already contains a fluorescent marker. The most relevant strategic change, however, concerns the placement of the promoter outside the cassette, that is, upstream from the FRT3 site. This position enables to strongly enrich the insertions arising from authentic RMCE.[40,42]

High expressers of type "A" (Fig. 3, top) that have been evaluated and enriched by FACS are subjected to cassette exchange. RMCE 1 causes the promoter-less cassette of the exchange vector, F3-*hygtk*-F, to replace the parental F3-*d2egfp*-F cassette whereby the former gets under the control of the preexisting CMV promoter, P^{cmv}. Exchange events are enriched in the presence of Hyg, and candidate cells ("B") are collected by FACS according to their loss of fluorescence. In RMCE 2, the promoter-less F3-*GTN*-F cassette is introduced to restore a fluorescent status ("A'"). Finally, enhanced green fluorescent protein (eGFP)-expressing cells are screened and their origin (targeted integration) is verified. Single clones, for which a unique retargetable site has permitted high and stable expression, are recovered and tested further for their exchangeability in the absence of drug selection. This procedure comprises a "clean" RMCE procedure (no selection gene, no auxiliary sequences besides the GOI) enabling the establishment of production cell lines for any GOI within a short period of time. To briefly summarize the central goals:

- *Purpose 1: select cells containing transcriptional hotspots before testing their RMCE competence.* Cells marked with a fluorescent reporter can be preselected such that only active transcription sites are subjected to RMCE 1. For cells with exchangeable sites, fluorescence is restored during RMCE 2, permitting the validation of expression stability. Candidate clones are processed to yield the master cell line(s), which are finally targeted by the GOI cassette. Recipients are FACS-isolated according to the loss of their fluorescence.
- *Purpose 2: enrich cells with a unique targetable site.* In the existing studies, the selection of clones with a unique targetable site relies on screening numerous individual clones derived by different gene transfer techniques.[43] In our strategy, RMCE-competent cells lose the fluorescence already in the first round of RMCE, indicating that all F3-*d2egfp*-F templates have been replaced by F3-*hygtk*-F, either in a single-copy manner or (less likely) in a multicopy manner. Cells for which some but not all copies of the parental construct have been exchanged will remain fluorescent and are, hence, FACS-eliminated.
- *Purpose 3: overcome interferences caused by extra integration events of the exchange plasmid.* In our pilot experiment, random integration of the F3-P^{cmv}*d2egfp*-F cassette contributed to cellular fluorescence. This is why we implemented a promoter trap strategy (Fig. 3), in which the promoter (P^{cmv}, residing between the F3 and F sites in the Fig. 1 pilot study) is placed upstream from the F3 site. This position guarantees that the exchange construct is expressed only upon RMCE-mediated integration. In rare events, an adventitious integration downstream from an endogenous promoter may happen, but, due to a rather weak fluorescence, these events will not seriously interfere with the expression of the targeted cassette.

Performance of the refined strategy

Establishment of parental clones for RMCE 1

In order to evaluate the expression potential of different integration sites under natural conditions and to avoid any consequences arising from spontaneous drug-resistance phenomena, we selected cells by two sorting steps after microporation of the linearized parental cassette (Fig. 4). Since low

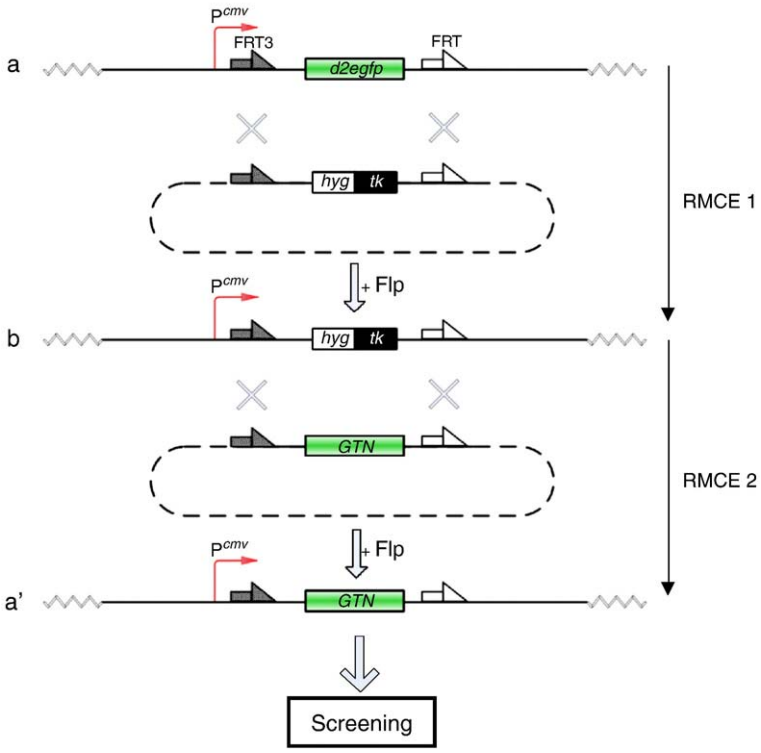

Fig. 3. An overview of a refined strategy based on the results in Fig. 2. A parental cassette encoding d2eGFP is microporated into CHO-K1 cells to create a pool of RMCE targets (a). Since the promoter is positioned externally from the parental cassette, that is, upstream from the FRT3 site, only the RMCE-mediated integration of the (promoter-less) exchange cassette will drive its transcription *via* the *cmv* promoter (P^{cmv}). High d2eGFP expressers are enriched by FACS multi-sorting. Following these steps, we used a promoter-less exchange cassette containing the *hygtk* fusion to replace the *d2egfp* gene (RMCE 1) and to create a pool of secondary RMCE targets (b). Successfully targeted cells can be collected by FACS since fluorescence will be abolished for B. In RMCE 2, a promoter-less exchange cassette containing the *egfp-tk-neo* (*GTN*) fusion is used to restore fluorescence. High expressers can again be screened *via* their fluorescence intensity. Finally, a subpopulation of clones from a′ bearing a unique targetable site with high and stable expression of eGFP can be chosen to develop production cell lines for various GOIs.

expressers may have growth advantages over high expressers, only the enriched population (designated "13/08-C1" in Fig. 4d) was subjected to RMCE 1, immediately after reaching confluence.

RMCE 1

After co-transfection of the exchange vector and an Flpo-expressing construct into 13/08-C1 cells, the transfectants were submitted to G418 selection (Fig. 5). Flpo is a novel mouse codon-optimized Flp variant, with recombination efficiencies similar to Cre.[44] Survivors from Hyg selection were almost exclusively found in the case of Flpo co-transfection (+Flp), rather than in the RMCE negative control (−Flp; Fig. 5b), confirming the targeted integration of the *hygtk* gene downstream from the *cmv* promoter. The persistence of few fluorescent cells

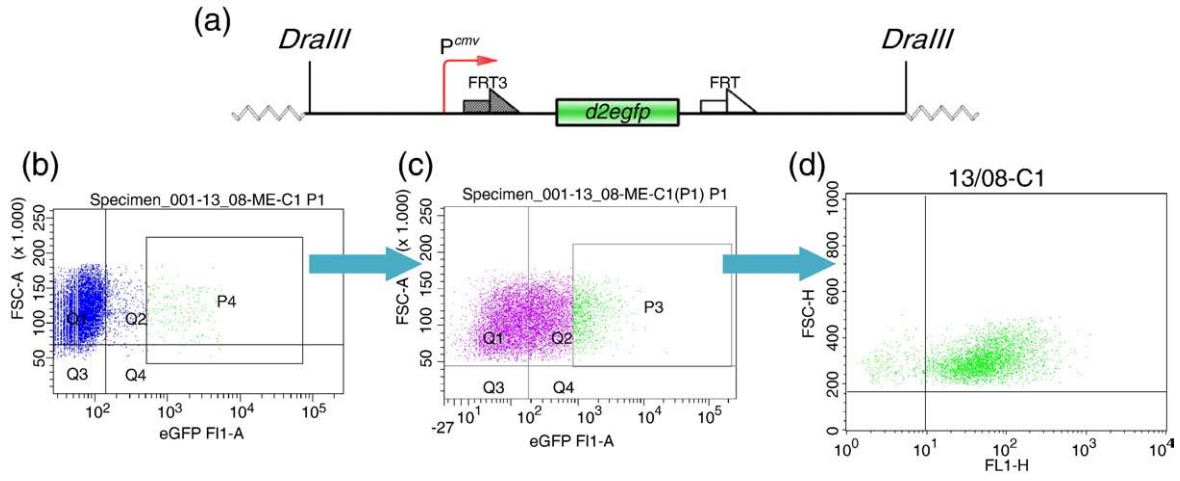

Fig. 4. Establishment of parental cells for RMCE. (a) Structure of the RMCE target: The parental vector contains a *d2egfp* gene flanked by FRT3 and FRT sites. The parental cassette is driven by a cassette-external promoter, P^{cmv}. (b) Pool of parental cells obtained from microporation of the linearized parental cassette into CHO-K1: Analysis was done 2 days post-microporation and 2% of the highest d2GFP-expressing cells were collected by FACS. (c and d) After 5 days, expanded cells revealed a wide range of expression levels and were sorted once more to recover 10% of the highest expressers, which were termed 13/08-C1 for the following experiments.

("H" population in "17/08-C1") can be ascribed to partially targeted integration of *hygtk* gene. While, in theory, the targeted integration of a single copy of the exchange cassette suffices to mediate Hyg resistance, the remainder can be identified by its residual fluorescence. PCR analyses on the Hyg-resistant cells, both on the highly and low-fluorescent subpopulations, "H" and "L", confirmed authentic exchange events. Only the expanded subpopulation L, termed "13/08-C1-L", was subjected to the next RMCE.

RMCE 2

(Fig. 6) exchanges the F3-*hygtk*-F cassette for F3-*GTN*-F (GTN, a *gfp-tk-neo* fusion), which could be selected in G418. High d2eGFP expressers were only found in the +Flp case but not in the −Flp control (Fig. 6b), for which the survival of very few colonies could be ascribed to the cryptic promoter of the amp gene (in the dashed part of vector sequences) initiating some expression of the GTN fusion.[45] Alternatively, threshold fluorescence might have been due to integration next to an endogenous promoter. Next, the +Flp fraction was dissected into a highly fluorescent (H) and a low-fluorescent to nonfluorescent population (L). In the +Flp case, authentic exchange events could be detected for situations H and L (but not for −Flp cells; Fig. 6c). For the L population, these events were ascribed to the integration of the GTN fusion, followed by silencing. Clonal cells were FACS-recovered from the H population and subjected to cassette exchange.

Characterization of single clones

Twenty-four single clones were isolated from the H population after two rounds of RMCE. In PCR

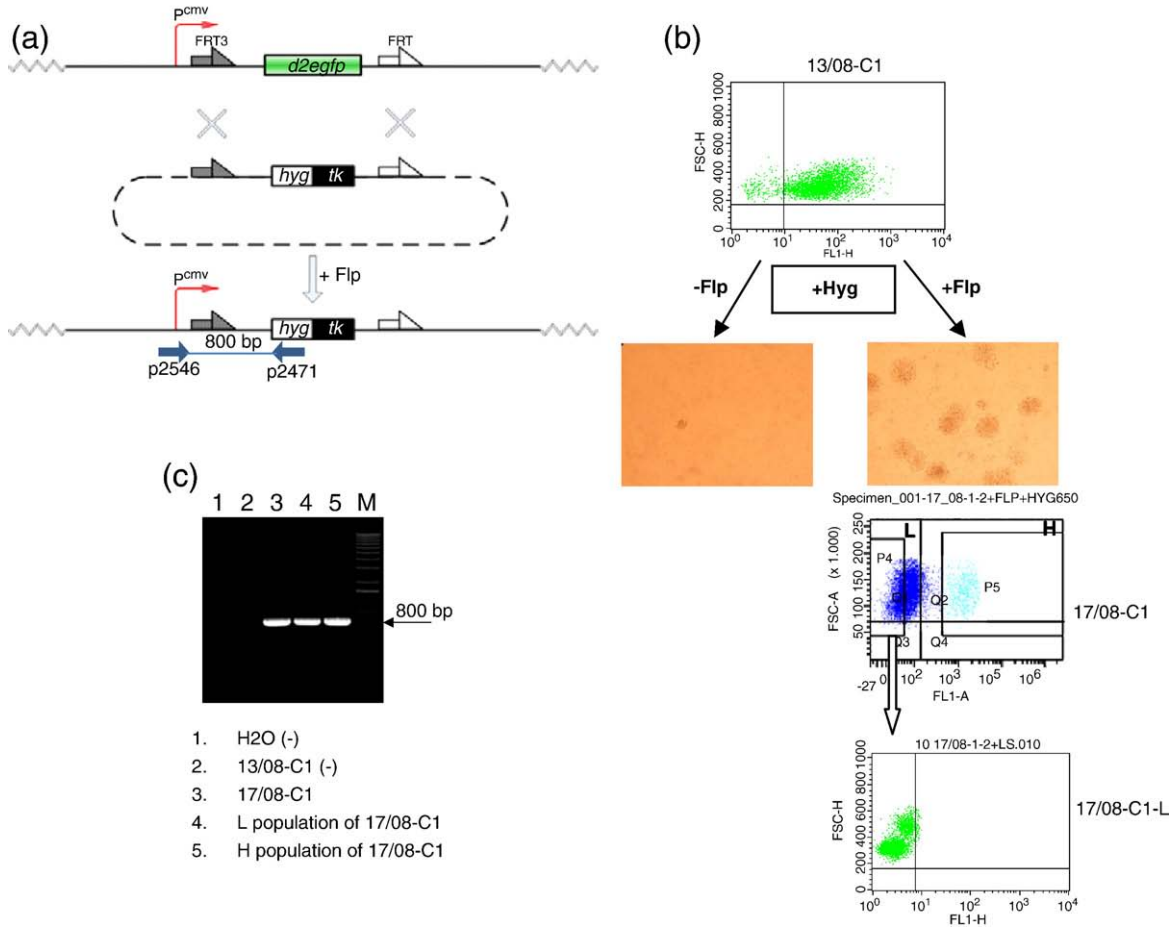

Fig. 5. RMCE 1 according to Fig. 3. (a) Outline: The exchange vector containing a promoter-less *hygtk* cassette was co-transfected into 13/08-C1 cells together with the *flpo-pac* vector. After transient selection with Puro for 2 days, the transfected cells were subjected to Hyg. (b) Enrichment of targeted cells for situation B (cf., Fig. 3) in the presence ("+Flp") or absence of *flpo-pac* ("−Flp"; negative control): After Hyg selection, multiple colonies could only be recovered for the "+Flp" case as expected. A significant population of nonfluorescent cells were detected by FACS analysis in the "+Flp" cells. The nonfluorescent (L) and the highly fluorescent (H) subpopulations were separated by FACS; the L population was then subjected to RMCE 2 (Fig. 6) as anticipated in the overview (Fig. 3). (c) Verification of authentic exchange events for the hygromycin-resistant population: PCR was performed on 17/08-C1 and two subpopulations (L and H) as indicated in (b). Negative controls were performed with 13/08-C1 cells and with H_2O. M: 1. 0-kb marker.

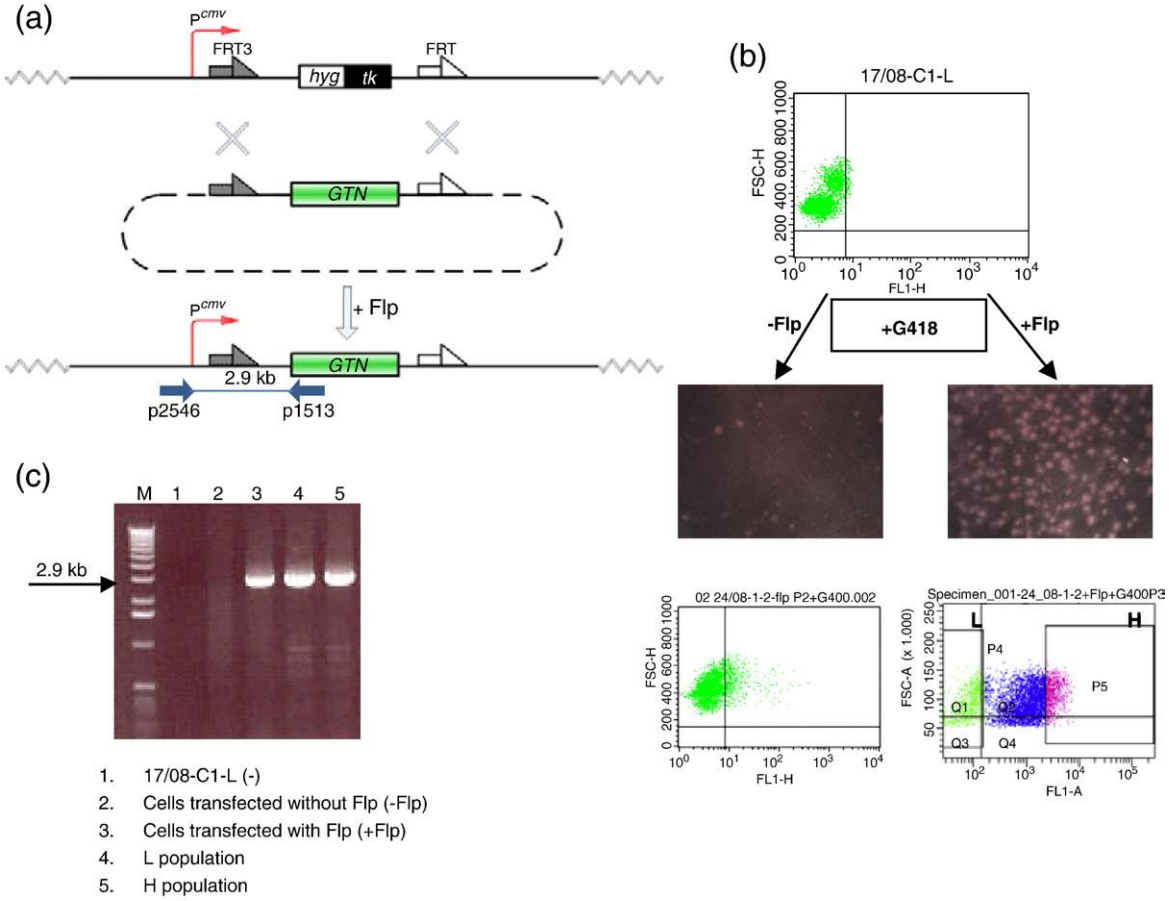

Fig. 6. RMCE 2. (a) Outline of the second exchange reaction: The exchange vector containing a promoter-less *egfp-tk-neo* (*GTN*) cassette was co-transfected, together with the *flpo-pac* construct, into "17/08-C1-L" cells. After transient selection with puromycin for 2 days, the transfected cells were subjected to G418 selection. (b) Enrichment of targeted cells: After G418 selection, a significant number of colonies could only be recovered in the "+Flp" experiment. This is confirmed by FACS analysis (bottom): highly fluorescent cells were only found for the "+Flp" cells but not the "−Flp" case. The "+Flp" cells were subdivided into a nonfluorescent population (L) and a highly fluorescent population (H). (c) Verification of authentic exchange events for the neomycin-resistant cells: Besides the L and H subpopulations, control PCRs were also performed on "17/08-C1-L" and "−Flp" cells. M: 1. 0-kb marker.

analyses, all of these proved an authentic exchange as for Fig. 6c (data not shown). These analyses were supplemented by Southern blots on NheI-digested genomic DNA of each clone (Fig. 7). Hybridization with a *gfp* probe yielded a defined 2. 7-kb band and confirmed an authentic RMCE-targeted *GTN* fusion gene for all but one ("C1-7") of the 24 isolated clones (Fig. 7c). In order to verify copy numbers and the distinct nature of the target sites (each copy was expected to yield a characteristic "bordering fragment"), we re-hybridized the stripped membrane with a *cmv* probe, which generated a unique but distinct band for all clones except "C1-2" and "C1-13" (Fig. 7b). Since the *cmv* promoter goes back to the parental cell (A in Fig. 3), these results confirmed that only clones with a unique targetable site have been enriched in the two rounds of RMCE and the subsequent selection steps. To summarize, except for clones C1-2 and C1-13 (multiple target sites) and clone C1-7 (additional randomly integrated exchange cassette), 21 (out of 24) clones showed a unique retargetable site with a single-copy gene integration event. To verify the stability of authentic targeted clones especially in the absence of selection pressure, we selected 9 (out of 21) variable clones based on the Southern blot analyses (Fig. 7b and c) and regularly analyzed them for their GFP expression (mean values of GFP-expressing population are plotted in Fig. 7d). Time-dependent overlay analyses were also performed and are embedded in Fig. 7d to underline the homogeneity of GPF expression for selected clones. Only clones with long-term stability and homogeneity were subjected to the remaining steps.

Transcriptional augmentation

S/MARs are able to guide the associated constructs to the machinery involved in histone turnover.[46] As a consequence, S/MAR-mediated transcriptional effects can be boosted by the addition

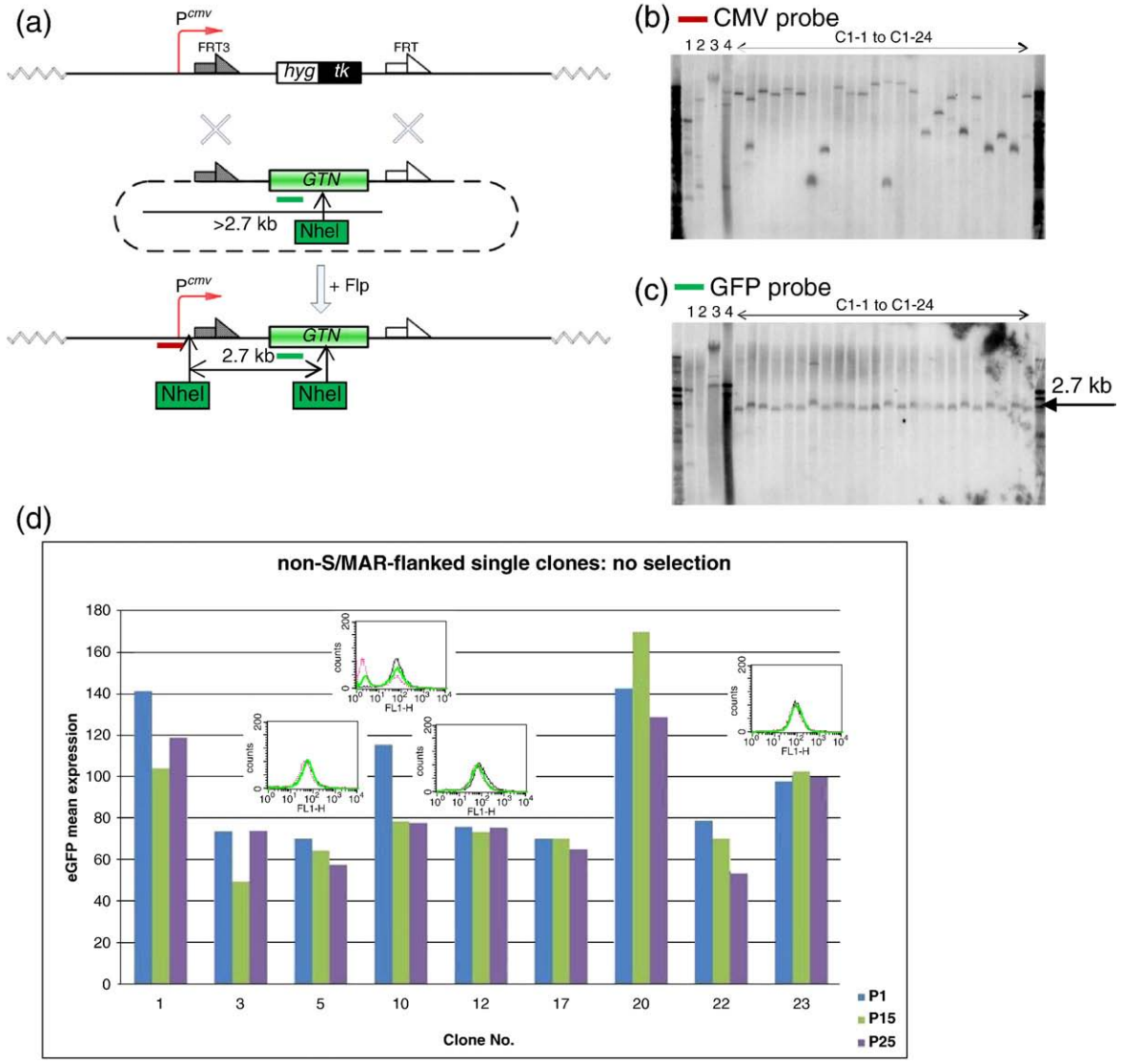

Fig. 7. Unique genomic targets with persistent long-term expression. (a) Strategy of Southern blot analyses: 24 isolated highly expressing (H) clones were subjected to NheI digestion, which cuts in the *cmv* promoter and the *GTN* fusion gene to yield a defined 2. 7-kb band for the targeted integration of the *GTN* cassette. (b) Hybridization with a *cmv* probe (red): A NheI cut in the *cmv* promoter and a cut at an unknown position in genomic DNA lead to a unique band for each integration event of the parental cassette. The number of band(s) indicates the copy number of integrated parental cassette(s). (c) Hybridization with a *gfp* probe (green): A defined 2. 7-kb band reveals targeted *GTN* cassette at the target site(s). An extra band indicates random integration(s) of exchange cassette(s). (d) Long-term stability and homogeneity of individual clones in the absence of selection pressure: the mean GFP expression of nine representative clones was analyzed at passages 1, 15 and 25 (over a 3-month culture). Overlay analysis of clone C1-5, C1-10, C1-12 and C1-23 is exemplified on top of the corresponding set of bars (passage 1, black curve; passage 15, green curve; passage 25, red curve).

of HDACis (such as NaBu or VPA), which give rise to high core histone acetylation levels. In our model study,[47] NaBu increased transcriptional levels <2× for S/MAR-free constructs while its effect was ~4× for the same test gene with one flanking S/MAR and ~30× in case the mentioned elements "E" and "W" constituted both domain borders (cf., Fig. 1 in Schlake *et al.*[47]). In the present series of experiments, NaBu treatment caused a significantly increased (9–11×) expression not only for the S/MAR case both also in the non-S/MAR situation (5–6×). The response to VPA was somewhat smaller in accord with its reduced HDACi potential (Fig. 8). The same properties were maintained in the final production strains.

Producer cells

As a substitute for the GOI, a model gene was introduced as part of the ultimate exchange cassette (Fig. 9a). After confluence, the nonfluorescent population of the recipient cells was immediately isolated by FACS counterselection, both for the +Flp and the –Flp case (see clone "C1-23" in Fig. 9b as an

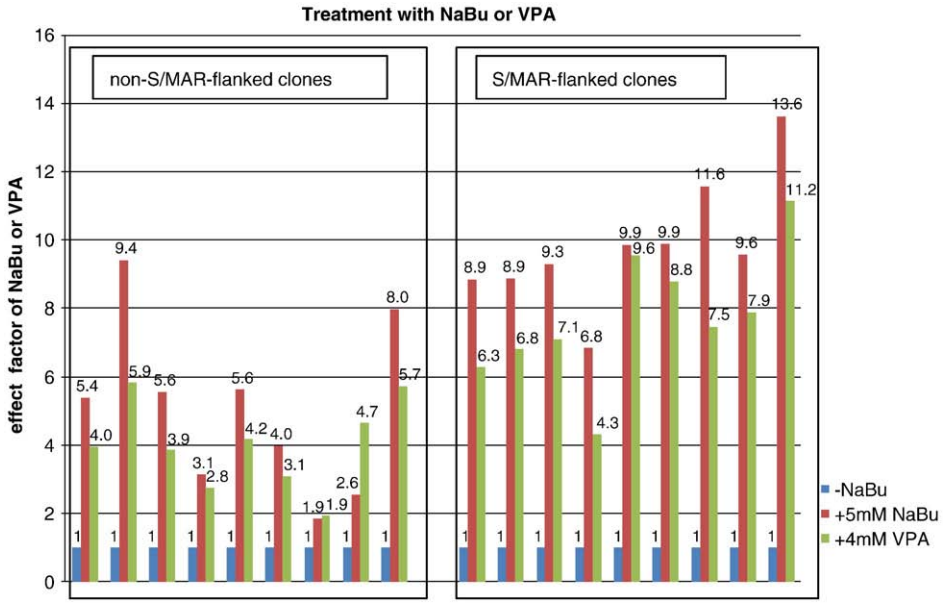

Fig. 8. The effect of HDACi on the transcription level of screened single clones. Each of nine randomly selected non-S/MAR- or S/MAR-flanked single clones was treated with the medium supplied with 5 mM NaBu or 4 mM VPA for 48 h. The cells were rinsed with PBS and then suspended for FACS analysis. The mean level of the GFP-expressing cells is taken as a measure and referenced to the expression level of untreated cells, which was arbitrarily set to 1. The resulting augmentation factor is indicated on the bars for both NaBu and VPA.

example). As expected for targeted integration, the vast majority of nonfluorescent cells arose in the +Flp population.

In order to identify efficient producer cells derived from three master lines, we sub-cloned 100 cells of each from the nonfluorescent population. For C1-23, 11 out of 100 were highly expressed; for "C2-1", 11 out of 100 were highly expressed; and for "C2-14", 9 out of 100 were highly expressed; the remainder showed either low or undetectable expression levels (data not shown). C1-23 is a master clone without S/MARs, while C2-1 and C2-14 are master clones for which S/MARs flank the GOI (Fig. 9c). In selected cases, control hybridizations were done with a GOI probe, which proved that virtually all clones showed targeted integration at a unique site (Fig. 9c). Only in one case of the C1-23 series was the authentic event accompanied by an extra, randomly integrated copy of the GOI.

In conclusion, our refined strategy is appropriate to derive, with high efficiency (>80%), master cell lines with a unique targetable site from randomly selected clones. These master cell lines show a stable and homogenous expression pattern with consistent retargetability in the presence and even in absence of selection pressure. The efficiency of isolating high production clones with only targeted integration of the GOI is around 10% in the absence of drug selection (Table 1) and close to 100% in case the GOI cassette is replaced by a GOI-IRES-*pac* cassette, and the process is supported by puromycin selection, which, however, does not comply with our primary goals (data not shown).

Discussion

Targeted integration of a GOI into a pre-identified transcriptional hotspot is a valuable approach to counteract the well-known unstable and unpredictable expression patterns caused by position effects. This concept has enabled a production platform in which the transgenic cell line contains a single-copy gene at a unique genomic location.

Here, we describe the benefits of a method consisting of two consecutive rounds of RMCE to isolate master clones with reusable hotspots that provide high and homogenous expression properties for any GOI. Relying on a simple promoter trap strategy and on FACS counterselection, these production cell lines can be derived in the absence of drug selection. Such a combination of complementary selection procedures proves to be largely superior to the traditional processes.

Master cells with a targetable transcriptional hotspot

For our tag-and-exchange strategy, candidate clones were randomly picked and characterized for the high and homogenous expression as well as the RMCE competence of the respective integration site. Since both of these characteristics do not necessarily correlate,[41] a large number of clones usually has to be screened to derive suitable candidates. Another complication relates to the ineffective negative selection principle in CHO cells, which leads to the enrichment of random integrations with a silenced selector gene, along with the correctly targeted

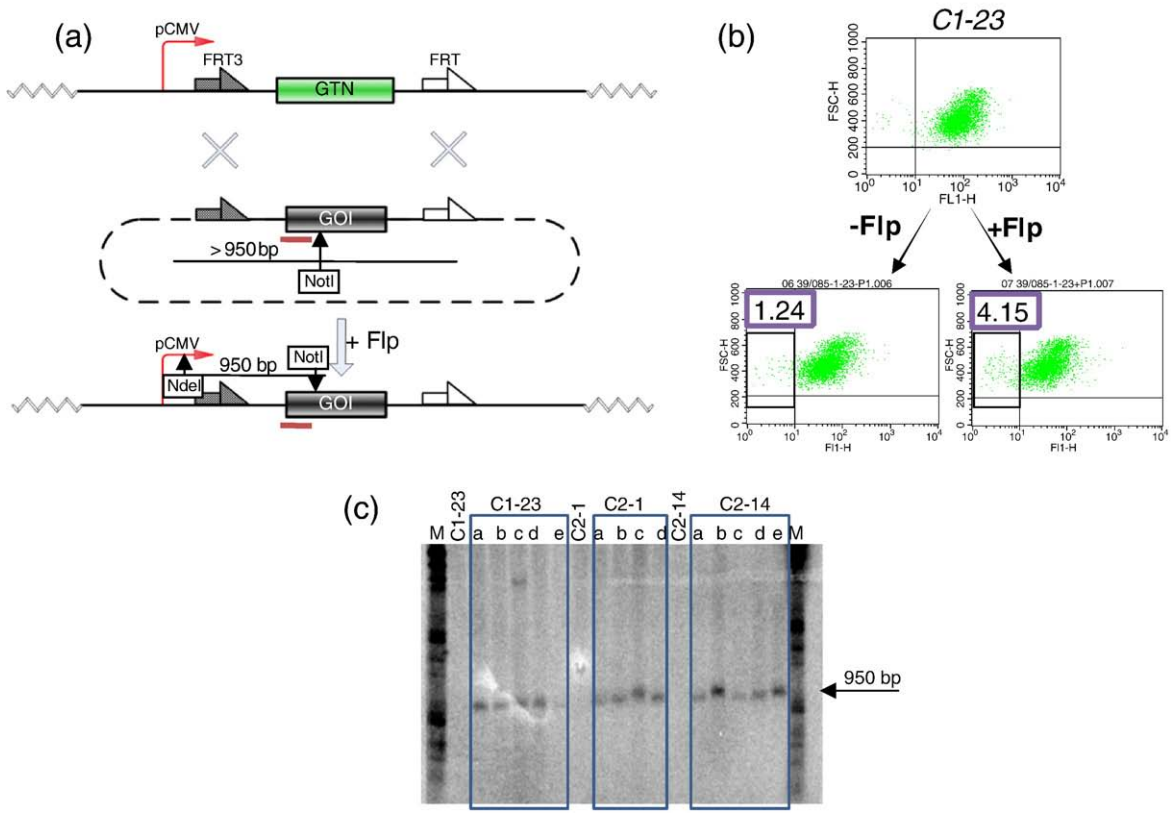

Fig. 9. (a) Development of cell lines for GOI production. Outline of RMCE to derive production cell lines from selected master cell clones: The GOI on the promoter-less exchange vector was used to replace the *GTN* cassette. After transient selection with puromycin for 2 days, the transfected cells were passaged into normal medium and monitored by FACS analysis, exemplified by clone C1-23 in (b). As expected, there were more nonfluorescent cells in the "+Flp" (4.15%) than in the "−Flp" experiment (1.24%). Single clones were isolated from the nonfluorescent population of the "+Flp" cells. (c) Southern blot analysis of randomly selected GOI expressers: NdeI- and NotI-restricted single clones were hybridized with a GOI probe. For our experiment, in the +Flp case, all clones revealed the RMCE-specific, 950-bp band as anticipated in (a). An extra band indicates the random integration of another GOI cassette (cf., lane c for C1-23).

events (Fig. 2). In our refined strategy, a pool of clones with RMCE-competent integration sites arose only after two successive rounds of RMCE. Members of these were screened and analyzed for single-copy gene integration (Fig. 7). In RMCE 1, the targeted cells lost fluorescence as the *d2egfp* cassette was replaced (Fig. 4). Cells for which some but not all copies of *d2egfp* have been replaced might have established Hyg resistance but would still be fluorescent and thereby excluded by FACS. Altogether, clones with a unique (or more than one) targetable site became strongly enriched in the two-step RMCE selection procedure. Since integration sites with both high expression level and established targetability had been prescreened, a largely reduced number of single clones were needed to derive a master cell line.

Table 1. Analysis of expressing clones derived from corresponding master lines by RMCE in the absence of selection

Master cell lines	GOI high expressers	Only RMCE-mediated integration	Clones with targeted integration (%)
C1-23	11/100	4/5	8.8
C2-1	11/100	4/4	11
C2-14	9/100	5/5	9

The efficiency of developing production cell lines

Nonspecific integration may seriously interfere with the function of a targeted gene construct. Since recombinase-mediated integration is restricted to a single locus whereas random integration can be widespread in the genome, correctly targeted cells may represent a minor population among the transfectants. As a consequence, a stringent selection procedure has to precede the ultimate screening of candidate master clones.

While coupling with a selection cassette would enrich both targeted and random integration events, the *neo*-complementation strategy used by others[18] necessarily leaves behind the *neo* selection marker, which causes unexpected and uncontrollable epigenetic modifications during cell line development.[22,23] In contrast, our promoter trap

strategy efficiently captures cells with only a targeted integration event (Fig. 7). At the clonal level, the GOI can ultimately be introduced at an efficiency of 10% in the absence of drug selection (Table 1). Among 14 characterized sub-clones from three different master cell lines, there was a single case for which random integration was detected (Fig. 9c). Although one might argue that our promoter trap strategy restricts the protocol to the promoter that has been chosen to establish the parental clones (Fig. 3a), the free promoter choice option in our pilot study does not provide a realistic advantage: the expression of a transgene always reflects a particular set of interactions between the promoter and its genomic environment. This was documented, for instance, by Feng *et al.*[48] and by Seibler[49] who showed that even the inversion of a transcription unit at a given locus can alter its expression properties.

Timeline

The preselection of high expressers by double sorting required 10 days (Fig. 4) while the selection of targeted cells in two RMCEs required 10–15 days (Figs. 5 and 6). The final step to screen clonal cells could be performed in another 20 days (Fig. 7). Therefore, 2 months are sufficient to identify targetable clones with both a unique integration site and a high expression level. Compared to the traditional "integration and selection" strategy (6 months)[4] or the conventional "tag-and-targeting" RMCE strategy (4 months),[18] our refined strategy is definitely more time efficient. For various GOIs, the production cell lines could be derived from established master lines within 1 month. Finally, since no drug selection is included, time and money will be saved in the downstream processing.

Transcriptional augmentation

Besides their role in structuring chromatin domains, S/MAR elements are involved in chromatin remodeling and the suppression of silencing phenomena that are ascribed to histone H3K9 trimethylation/DNA methylation or histone deacetylation.[28] We investigated the effect of S/MARs at the borders of the target cassette in order to find out if it is possible to improve clones arising from our selection scheme with regard to transcriptional augmentation[28] and long-term stability. Elements that have proven their performance before[47] were the 2. 2-kb element E (from the human IFN-β gene; upstream) and the unrelated 1. 3-kb element W (from the first intron of the potato leaf stem-specific protein ST-LS1; downstream). Since these elements contain a minimum of common sequences, the danger of their cross-recombination could be kept low.[28,36,50]

In our pilot experiment, corresponding setups (presence or absence of S/MAR boundaries in Fig. 1c and f) have indicated that S/MAR-flanked cassettes can give rise to a significantly larger proportion of cells with high d2eGFP expression. This phenomenon may depend on several facts:

- S/MARs function as bordering elements to alleviate silencing that would result from heterochromatization;[28]
- S/MARs increase transfection-mediated integration events for linearized templates, possibly due to their effect on single-stranded DNA end formation;[51,52]
- based on their DNA-strand separation potential, S/MARs are recombinogenic elements that may support the rate of RMCE. This may have caused the higher proportion of Ganc-resistant cells in the Fig. 1 experiment.

Since our refined strategy strongly enriches for high and stable expressers, the non-S/MAR-flanked cassettes have an expression profile resembling the S/MAR case. This effect may be associated with the enrichment process, which tends to prefer transgenes in a wider S/MAR context. The fact that treatment of individual clones with HDACis boosts transcription[46,47] is certainly in accord with such a model: S/MARs assemble the histone acetylation machinery and direct its activity to the interior of the respective gene domain to cause its pre-activation.[53]

Current status and outlook

The RMCE protocol we have detailed here relies on the most efficient combination of FRT sites (F and F3) originally introduced by Schlake *et al.*[16] In this combination, the F3 site meets all of the following criteria:

1. the 8-bp spacer sequence has an AT content of >75% (88% for F3) to facilitate the double-reciprocal recombination reaction between identical FRTs;
2. the two extreme positions 1 and 8 remain unchanged to enable an optimal Flp-binding potential;
3. an uninterrupted 5′ polypyrimidine tract extends from the spacer into the adjacent 13-bp repeat;
4. if criteria 1–3 are met, the mutual recognition of site mutants (F′ × F′) is as effective as for the wild type (F × F). If, in addition, the 48-bp F and F′ sites differ in 4/8 spacer positions, there is a close to 100% site discrimination at the transient state and no cross-recombination at all if the target has been genomically anchored. Apparently, these favorable properties of Flp RMCE are not shared by the Cre-LoxP system, which relies on 34-bp lox sites.[54,55]

Use of the F3 × F combination (rather than the F5 × F set) may account for the fact that our system performs well in the absence of rigorous measures

such as promoter and ATG traps that had to be applied as prerequisites for the RMCE-dependent expression of drug-selection genes, especially the *neo*^{r} cassette. It may be of interest that, meanwhile, we have seven different mutants available (F′) that can be used in combination with the wild-type site (F) or with one another. This repertoire will enable multiplexing approaches,[56] as it permits the parallel establishment of several independent and non-cross-interacting genomic targets (S. Turan, unpublished results). If present in a master cell line, this will allow the parallel expression of two different protein subunits, for instance, for antibody production. Such a strategy might circumvent the barely predictable performance of bicistronic systems, which depend on both the nature of the IRES element and the cistrons.

The use of well-characterized genomic *loci* and cellular promoters is one of the goals, which is amenable by tag-and-exchange strategies in which the tag is set by homologous recombination (HR). The low efficiency of HR can nowadays be overcome by zinc finger nucleases, which introduce a double-strand DNA break at the desired location[57] enabling HR with high efficiency and specificity. If HR is used to introduce target cassette(s) flanked by set(s) of heterospecific FRTs, our selection procedures can be applied to facilitate subsequent exchange reactions in the absence of drug selection. It also opens novel options for generating ES and induced pluripotent stem cells and for the deliberate deletion of several transgenes or elements without the risk of multiple site interactions (see the 'flrting' concepts described in Ref. 35).

Materials and Methods

Plasmids

Construction of the plasmid F3-*hygtk*-F has been described previously.[16] The *hygtk* gene in F3-*hygtk*-F is a fusion gene coding hygromycin B phosphotransferase and HSV thymidine kinase under the control of the HSV-*tk* promoter. For the construction of plasmid EF3-*hygtk*-FW, the amplified F3-*hygtk*-F cassette was restricted with SalI/SpeI and inserted into the backbone of plasmid E-*SGTN*-W to replace the *SGTN* unit.

Exchange vector F3-P^{cmv}*d2egfp*-F was constructed by inserting the amplified P^{cmv}*d2egfp* fragment coding d2eGFP under control of the cytomegalovirus (*cmv*) promoter into KpnI/SpeI-digested plasmid F3MCSF (Clontech, California, USA). The homologies between the backbone of parental vector (F3-*hygtk*-F) and that of exchange vector (F3-P^{cmv}*d2egfp*-F) were minimized in order to distinguish targeted integration from random integration by PCR analyses.

Plasmid P^{cmv}F3-*d2egfp*-F was obtained by ligating the NheI/SalI-restricted F3-*d2egfp*-F fragment into NheI/SalI-digested plasmid P^{cmv}*d2egfp*-basic (Clontech).

Plasmid EP^{cmv}F3-*d2egfp*-FW was derived in a way analogous to plasmid EF3-*hygtk*-FW by replacing the *hygtk* fusion with P^{cmv}F3-*d2egfp*-F amplified from plasmid P^{cmv}F3-*d2egfp*-F.

Plasmid F3-*hygtk*-F(w/o) was derived from F3-*hygtk*-F, in which the *tk* promoter was deleted by MluI/XhoI digestion, filling and ligation.

Plasmid F3-*GTN*-F was derived from plasmid F3-*SGTN*-F,[58] by deleting the *sv40* promoter upstream of *GTN* fusion encoding eGFP (d2eGFP), thymidine kinase (TK) and neomycin (Neo).

Plasmid F3-*GOI-IRES-pac*-F was derived in a way analogous to plasmid F3-P^{cmv}*d2egfp*-F but with the use of a bicistronic construct *GOI-IRES-pac* in place of P^{cmv}*d2egfp*.

Cell culture

The CHO-K1 cell line (European Collection of Cell Cultures No. 85051005) was cultured in Dulbecco's modified Eagle's medium (DMEM)/Nut. Mix F12 (HAM) medium (1:1) supplemented with 10% fetal calf serum, 20 mM glutamine, 60 μg penicillin/ml, and 100 μg streptomycin/ml.[59] Cells were incubated at 37 °C with 5% CO_2 in Labotect CO_2 incubator C2000 (Labor-Technik-Goettingen). Cells were passaged 3 or 4 days after reaching 90% confluence on a six-well plate or a little flask.

Gene transfer

Electroporation

We collected 1E6 logarithmically growing, semi-confluent cells by trypsinization and resuspended them in 0.7 ml prechilled DMEM/HAM medium together with 3 μg linearized DNA. The cell–DNA mixture was pulsed at 360 V, 800 μF capacity and infinite resistance with a Biorad Gene Pulser and Pulse Controller (Biorad, California, USA). The electroporated cells were plated into a 10-cm plate containing 10 ml DMEM/HAM medium.

Microporation

At later stages (refined strategy), the more recent microporation technology has replaced electroporation. With the use of pipette-tip electrodes, the method provides uniform electrical pulses for 10 - to 100-μl samples, eliminating harmful side effects such as pH variation, temperature increase, turbulence and metal ion generation. This results in an improved transfection efficiency and cell viability. We collected 5E5 logarithmically growing, semi-confluent cells by trypsinization and resuspended them in 100 μl Buffer R with 5 μg linearized DNA. The cell–DNA mixture was aspirated using Microporator 100 μl pipette and microporated at 1620 V with MicroPorator MP-100 (NanoEnTek Inc., Seoul, Korea). The pulse width was 10 ms, and the sample was pulsed 3 times. The microporated cells were seeded into a 10-cm plate containing 10 ml DMEM/HAM medium.

Transfections for RMCE

One day prior to transfection, cells (1.2E5/well) were seeded on a six-well plate. Four hours before transfection, the medium was refreshed with DMEM/HAM medium. Cells were co-transfected with 3 μg exchange vector and 1 μg Flp expression vector (containing either the *flpe-pac* or the *flpo-pac* construct) according to the Metafectene protocol (Biontex, Germany). One day post-transfection, the cells were treated with 2.5 μg/ml puromycin for 48 h.

For stable selection, phosphate-buffered saline (PBS)-rinsed cells were then passaged into media supplemented with corresponding antibiotic.

Negative control

Mimicking RMCE transfection, the neutral vector BSpac-Δp[60] only encoding puromycin was used in place of the Flp expression for the control transfection: If integration happened, it was independent on Flp recombinase.

Polymerase chain reaction

Genomic DNA was harvested from the cell lysate with NaAc/EtOH. For PCR, amplification was done with the Expand Long Template Enzyme Mix (Roche, Germany) according to the manufacturer's manual. A standard amplification program for the Thermo-Cycler (Biometra, Germany) proceeded as follows: pre-denaturation step: 94 °C, 3 min; denaturation step: 94 °C, 30 s; annealing: 58 °C, 1 min; elongation: 68 °C, 2 min; cycling: 30 cycles; termination: 4 °C. The primers used are listed below:

p2494: 5′-CAATTAATGTGAGTTAGCTC-3′
p2471: 5′-GTCGCGGTGAGTTCAGGCTT-3′
p1230: 5′-GACGACGCGGCCGCTATATGTTATTTTCCAC-CATATTGC-3′
p2484: 5′-CTCGAGATCTGAGTCCGGTAGCGCTAGCG-GATCTGACGGTT-3′
pGFP6: 5′-ACACGCTGAACTTGTGGCCGTTCACGTCGC-3′
p2546: 5′-CGATCGGTGCGGGCCTCTTCGCTATTAGG-CCAGC-3′
p1513: 5′-GAGAACCTGCGTGCAATCCATC-3′

Southern blot analysis

Genomic DNA (~10 μg) was digested overnight with an appropriate enzyme and separated by electrophoresis on a 0.8% agarose gel in 1× TAE buffer. DNA was transferred to a positively charged nylon membrane (Amersham, UK) in 0.4 M NaOH. The membrane was then neutralized and baked at 80 °C for 2 h. Pre-hybridizations were done in buffer containing 0.5 M $NaxHyPO_4$ (1 M Na_2HPO_4 and 1 M NaH_2PO_4 mixed at a ratio of 4:1), pH 7.2, 7% SDS and 2 mM ethylenediaminetetraacetic acid at 65 °C in an incubator for at least 30 min. The probe was labeled with 5 μl of α[^{32}P]dCTP and amplified with the "Redi-Prime DNA labeling system" (Amersham, UK). Hybridization with the labeled probe was performed overnight. After washing, the membrane was exposed in a Molecular Dynamics Exposition Cassette (Amersham, UK) and scanned by a Phosphor-Imager (Molecular Dynamics).

Flow cytometry

Scanning (FACSCalibur)

The expression of eGFP was analyzed by a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA, USA). Confluent cells grown on a six-well plate were collected by trypsinization. The cellular pellet was resuspended in 500 μl prechilled PBS containing 2% deactivated fetal calf serum and 10 μg/ml propidium iodide (PI). The excitation wavelength for eGFP was 488 nm and emission was detected at 488 nm (on Fl-1). The PI emission at 620 nm was detected on Fl-3. Dead cells were excluded *via* an FSC-H *versus* Fl-3 dot plot. The GFP expression was evaluated in an FSC-H *versus* Fl-1 dot plot on the live cells. We collected 1E5 events. Data were acquired and analyzed by CellQuest™ Pro.

Sorting (FACSAria)

The single cells or cell population expressing eGFP were separated by a fluorescence-activated cell sorter (FACSAria; Becton Dickinson). The suspended cells were passed through a 40-μm gauze to exclude clumped cells. eGFP-expressing cells can be detected and sorted at wavelength of 488 nm on Fl1-A. PI emission was detected at a wavelength of 620 nm on Fl3-A. Doublets were excluded *via* an FSC-H *versus* FSC-A dot plot. The dead cells were excluded *via* an FSC-A *versus* Fl3-A dot plot. The sorting gate was the combination of the live cell gate, the doublet discrimination gate and the dot plot gate on FSC-A *versus* Fl1-A. The sorted cells were incubated several days in the medium with 5 μg/ml gentamycin. Data were acquired and analyzed by FACSDiva software.

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