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Mini-Review

Advances in drug-free acquisition of gene-engineered mammalian cells: A mini-review

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ABSTRACT

Transfection of mammalian cells with exogenous DNA such as plasmids is a valuable technique for exploring the biological functions of a gene of interest. Traditional gene delivery approaches using non-viral vectors employ drug resistance genes as selectable markers, leaving a genetic 'scar' that can interfere with cell survival and with the analysis of the resulting phenotype. Furthermore, it is difficult to perform gene transfer in multiple-drug resistant cells. Fluorescence-activated cell sorting (FACS) is a powerful method for enrichment of transfected cells under drug-free conditions, but the equipment used for FACS is very expensive and sometimes laborious to operate. Therefore, novel methods are required for obtaining stable transfectants that do not depend on drug selection and can be performed in a more convenient and easier manner. In this review, we summarize the achievements of the drug-free acquisition of gene-engineered mammalian cells. Special attention is focused on the recent advances in the acquisition of mutated cells by using CRISPR/Cas9-based genome editing, a novel and recently developed technology, under drug-free conditions.

KEYWORDS: selective drug, FACS, transfection, genome editing, transfectant, CRISPR/Cas9, targeted toxin.

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INTRODUCTION

Gene (DNA) delivery to cultured cells, which is called 'transfection,' is widely used to study the function and regulation of genes of interest (GOI) in a variety of cell types. Generally, gene expression in mammalian cells can be achieved either by transient or stable transfection with expression vectors that carry the GOI, the expression of which is controlled by a constitutive or tissue-specific promoter [1, 2]. The expression vectors are largely divided into two groups, namely non-viral and viral vectors. The former, which are based on plasmids, have been more commonly used for transfection experiments than the latter, which are based on adenoviruses, adeno-associated viruses, or lentiviruses.

The transfection method that is extensively used for transfer of GOI into mammalian cells employs co-transfection with a non-viral vector carrying a selectable drug marker like a neomycin resistance gene (*neo*) and an expression vector carrying the GOI, or with an all-in-one vector conferring the simultaneous expression of both the selectable marker gene and the GOI, as depicted in Fig. 1. After transfection for 24-72 h, the cells comprise of two fractions, namely transfected and untransfected cells. The former cells further include two types of cells, namely 'transiently transfected cells' that express the transgene that is not integrated into host genome, and 'stably transfected cells', or 'stable transfectants' that express the transgene integrated into the host

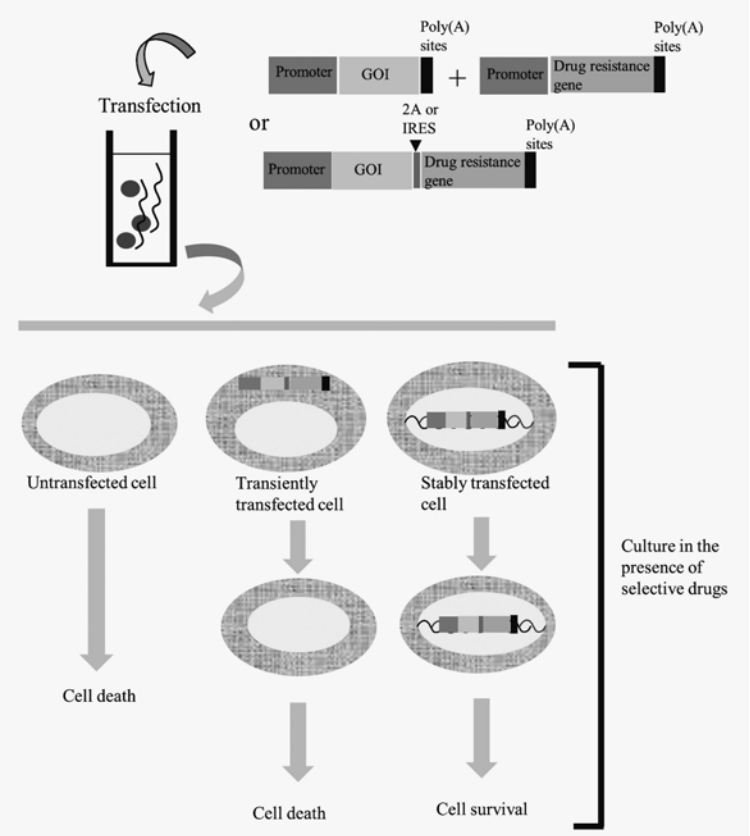

Fig. 1. The traditional transfection method for the isolation of stable transfectants. In case of transfecting mammalian cells with non-viral vectors, co-transfection with two constructs (GOI or drug-resistance gene expression vector) or transfection with a single construct conferring the expression of both the GOI and the drug resistance gene is employed. 2A, self-cleaving sequence; IRES, internal ribosomal entry site.

genome. Transient expression without a selectable drug allows functional analysis of the transfected gene, but may cause some problems in interpreting the results, due to the presence of a large fraction of untransfected cells. For obtaining stable transfectants and eliminating the transiently transfected cells, the trasfected cells need to be continuously cultivated in the presence of selective drugs. However, this method of selection is a long-term process and may have adverse effects on host cell functions, probably due to integration of the exogenous DNA into loci related to cell survival. Furthermore, it is difficult to perform gene transfer in multiple-drug resistant cells. Notably, the selective drugs that are used differ in their effectiveness for killing untransfected cells. Thus, researchers have to determine the optimal concentrations of the selective drugs that cause cell death of normal (but not transfected) cells, which again depends on the type of cells used. For example, in our experience [3] for porcine fetal fibroblastic cells (PEFs), treatment with 2 μg/mL of puromycin, an aminonucleoside antibiotic, was most effective; the time required for inducing death in half of the cells was only 1.5-1.8 days. Treatment with 40 μg/mL of hygromycin B (an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus*) and 8 μg/ml of blasticidin S (a peptidyl nucleoside antibiotic isolated from *Streptomyces griseochromogenes*) required approximately 3 days and that with 400 μg/mL of G418 required 3-4 days. With 800 μg/mL of zeocin, a member of the bleomycin/phleomycin family, isolated from *Streptomyces*, 4-5 days were needed for half of the cells to die.

The most desirable manner for DNA-mediated gene delivery to cultured cells may involve integration of the transgene into a defined locus of the host genome as opposed to integration into unrelated

loci (called as 'random integration'). In this case, the insertion of a selectable marker gene is not desirable. In this context, the knock-in (KI) technology [4, 5], which was originally developed by using a modified method of the traditional gene targeting system, appears to meet this requirement. Recently, gene knockout has been successfully achieved by employing the newly developed genome editing techniques including the zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) (CRISPR/Cas9) techniques [6, 7]. The first two methods cause mutations at the desired locus in the absence of donor DNA. ZFN and TALEN can introduce double-stranded breaks (DSBs) at the target site in the host chromosome, which are repaired by nonhomologous-end-joining (NHEJ). The NHEJ-based repair process generates an insertion or deletion of very few nucleotides, called an 'indel mutation', and causes a frame-shift that disables the encoded proteins or forms premature stop codons, thus generating a loss-of-function allele. In CRISPR/Cas9-based genome editing, a guide RNA (gRNA) that can bind to specific sites of the chromosomal DNA is required, along with an endonuclease called Cas9 [8-11]. Using these three genome-editing systems, successful genome modifications have been achieved in various organisms, including mice, rats, rabbits, pigs, bovine, monkeys, and humans [6, 7]. Due to the ease of design and assembly, and the availability of target sites, CRISPR/Cas9 is employed more frequently than ZFN and TALEN for the production of genetically modified animals [6, 7]. Additionally, it is possible to perform KI of a desired sequence using the CRISPR/Cas9 genome editing system [12-15]. CRISPR/Cas9-mediated disruption of a target gene is simple and rapid, because it does not require targeted vector construction and long-term selection of transfected cells in the presence of selective drugs. For instance, when we transfected PEFs using donor DNA (corresponding to the endogenous gene; containing mutations to stop protein synthesis of a target gene; spanning ~800 bp) with a humanized (h) Cas9 expression vector and a gRNA expression vector, followed by treatment with a reagent that inhibits the survival of cells lacking the targeted protein, several surviving colonies showed the KI alleles [16].

In this review, we summarize the previously developed drug-free systems for the enrichment of genetically modified cells, and describe a recent strategy to achieve desirable mutations in a defined, targeted locus without the use of selective drugs.

1. Previously developed drug-free isolation systems for enrichment of genetically modified cells

1.1. FACS/magnetic affinity cell sorting (MACS)-based isolation of stable transfectants

FACS is a method based on the collection of specific cells with fluorescent protein expression or with fluorescent markers bound to their surface [17, 18]. For this, cells are first transfected with a construct carrying a GOI expression unit and a unit containing a gene for a fluorescent marker such as enhanced green fluorescent protein (EGFP), and then subjected to FACS to obtain stable EGFP-expressing cells. By this treatment, it is possible to obtain several single-cell derived clones with variable levels of EGFP expression and once obtained, these cells can be grown in the absence of selective drugs [19]. It is highly possible that the GOI is also actively expressed in these isolated clones and its expression may be constant over prolonged periods. This system is indeed powerful for obtaining stable transfectants without the use of selectable growth media, but the FACS machine itself is very expensive and sometimes laborious to operate.

MACS is developed to enrich cells that express a specific cell-surface protein and is well-known as a commercially available approach that is much cheaper and easier to operate compared to the FACS-based approach [20-23]. In this case, antibodies capable of binding to a specific cell-surface molecule are used. Cells transfected with a DNA construct carrying the gene encoding the specific molecule are incubated with antibodies conjugated to biotin for affinity purification using a solid streptavidin-conjugated matrix, which is typically magnetic beads [24]. Only cells that express the specific molecule on their surface will be captured when placing the tubes with the cell suspension in the presence of a magnet. The cells bound to the magnetic beads collect on the side/bottom of the tube where the magnetic field is applied, and the non-adherent untransfected cells can be removed by gentle pipetting. This system, called immunomagnetic selection, is supported by a number of widely used commercial systems, and is indeed powerful for obtaining stable transfectants

without the use of selectable growth media, similar to the FACS-based cell sorting system. However, the target molecules for this system are limited to those expressed on the cell surface. In addition, specific antibodies that react with the target molecule are required. According to Siebenkotten and Behrens-Jung [25], the co-expression of a gene encoding the CD4 surface protein (with a truncated cytoplasmic domain and therefore cannot trigger signal transduction) along with the GOI is recommended, since commercially available anti-CD4 antibodies have been proven to be functional.

1.2. Isolation of stable transfectants by rescuing defective metabolic pathway

Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase (DHFR) expression do not survive without added nucleosides. However, these cells can survive in normal medium, if they are stably transfected with a DNA construct carrying the DHFR gene [26], as DFHR-expressing cells can synthesize the required nucleosides for survival. Similarly, cells lacking hypoxanthine phosphoribosyltransferase (HPRT) expression cannot survive in the specific medium called HAT medium, which contains hypoxanthine/amethopterin/thymidine; however, they can survive upon addition of nucleosides to the medium. When these cells are stably transfected with a construct containing the HPRT gene, they can survive in HAT media, whereas untransfected cells will be killed [27]. Thus, introduction of a construct that can express both the DHFR gene and the GOI in DHFR-deficient cells can be useful for generating stable transfectants. An additional advantage of using DHFR as a marker is the gene amplification of DHFR upon exposure to increasing doses of methotrexate and the simultaneous expression of transfected DNA (GOI), resulting in multiple copies of the transgenes in transfected cells [28]. This system will be helpful for the researchers who wish to obtain a transgene-high-expressor for producing large amounts of recombinant proteins *in vitro*. Unfortunately, this system is only limited to a few cell lines that are known to lack expression of genes required for cell survival. In this context, CRISPR/Cas9-based creation of several mutant cell lines like DHFR- or HPRT-deficient cells will be worthwhile, since they could be used for functional analysis of GOI in various fields of study.

2. Novel strategies for the drug-free selection of transfectants

2.1. Isolation of stable transfectants using the targeted toxin technology

The term ‘targeted toxins’ means hybrid molecules comprised of the ribosome-inactivating protein, saporin (SAP) [29] and a target molecule that recognizes a cell-specific marker. Several types of these targeted toxins are now commercially available from Advanced Targeting Systems, Inc. When targeted toxins are administered to cells of interest, they bind to cells that express the target molecule, resulting in cell death through inactivation of protein synthesis by the ribosomes. If cells do not express the target molecule, targeted toxins cannot bind to the cells, allowing them to survive. Our group has applied this system to enrich genetically modified cells without the need for selective drugs [30, 31]. The principle of the ‘targeted toxin technology’ is schematically shown in Fig. 2. This novel system requires the introduction of *Clostridium perfringens*-derived endo-β-galactosidase C (EndoGalC) gene expression construct that confers the digestion of a cell-surface carbohydrate moiety called α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R) [32, 33]. This moiety is expressed on almost all mammalian cells, except on those from humans and Old World monkeys [34-37]. The α-Gal epitope is synthesized via α-1,3-galactosyltransferase (α-GalT) localized on the cell surface [38]. In addition, the absence of an α-Gal epitope can be easily monitored by staining the cells with *Bandeiraea simplicifolia* isolectin-B4 (IB4), a lectin that specifically binds to the α-Gal epitope [34]. Notably, cloned piglets lacking the expression of α-GalT that are produced by traditional gene targeting are viable, suggesting no requirement of α-GalT expression for survival [39, 40]. As shown in Fig. 2, cells are first transfected with a construct that confers the simultaneous expression of both EndoGalC and the GOI. Four to five days after transfection (at which point the exogenous DNA is considered to have integrated into the host genome), cells are harvested by trypsinization and immediately treated with IB4 conjugated to SAP (IB4SAP; commercially available from Advanced Targeting Systems, Inc.) for a short period (at 37 °C for 2 h). This treatment can eliminate the unwanted cells, including those that are untransfected and those with only weak

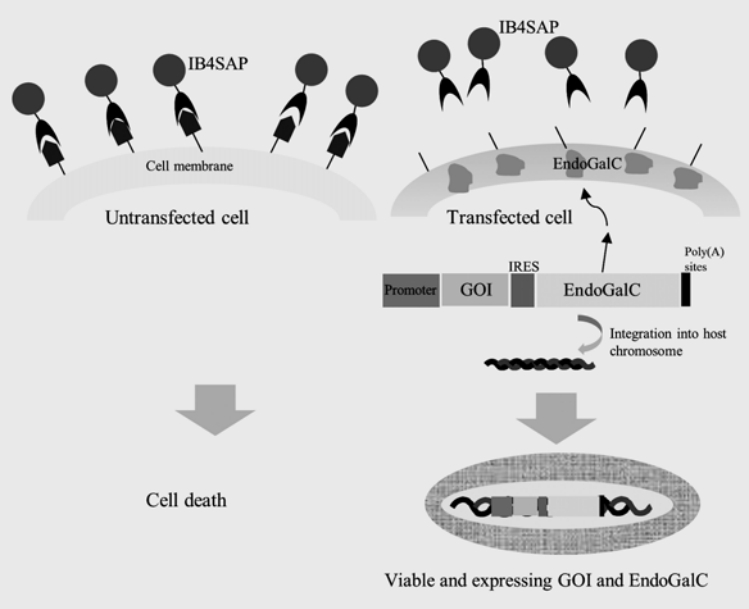

Fig. 2. Targeted toxin-based drug-free isolation of stable transfectants. Transient expression of EndoGalC from a single vector conferring expression of both the GOI and EndoGalC should result in resistance to isolectin BS-I-B_4 conjugated to saporin (IB4SAP) in transfected cells, causing death of α-Gal epitope-expressing untransfected cells. Concomitantly, such an introduced vector has the chance to get integrated into the host chromosome. Thus, the surviving cell population is expected to express the GOI and EndoGalC.

expression of the α-Gal epitope. These treated cells are subsequently cultured in normal media (without selective drugs) to permit colony formation. The surviving cells should not express the α-Gal epitope on their surface due to EndoGalC expression, which is derived from the chromosomally integrated transgenes. They should also express the GOI strongly since the expression of both EndoGalC and GOI is controlled by the same ubiquitous promoter. The demerit of this system may be that it requires the use of the EndoGalC gene that is not commercially available yet, and is applicable only to cells expressing the α-Gal epitope. Acquisition of stable human/Old World monkey-derived cells expressing whole porcine α-GalT cDNA by transfection with an α-GalT expression plasmid may overcome such limitations.

The *piggyBac* (PB) system, derived from the cabbage looper moth *Trichoplusia ni* [41], was developed as an efficient gene delivery tool for various mammalian cells [42, 43]. In this system, a transgene inserted between inverted repeat elements, called 'ITRs' in the PB transposon is excised and integrated into the host genome via transposition activity provided by the PB transposase enzyme encoded on a separate vector. Therefore, only an expression unit surrounded by the ITRs is integrated into the host genome upon transgene integration *via a* PB-specific TTAA site. Furthermore, the chromosomally integrated transgenes can be excised from the transfectants by the introduction and expression of the PB transposase expression vector [44, 45]. This PB-based gene delivery system is now used in various fields of study including *in vivo* gene transfer in mice [46] and for the generation of inducible pluripotent stem (iPS) cells [47, 48]. We have employed this PB-based gene delivery for acquiring stable transfectants more efficiently by using it along with the targeted toxin technology [49]. This system is based on the simultaneous expression of EndoGalC and PB transposase encoded on a single vector, called pTransIEnd. We demonstrated the utility of this unique system by using PEFs. The PEFs were co-transfected with pTransIEnd and a PB transposon vector carrying the GOI (i.e., EGFP cDNA or lacZ gene) and after two or three days of transfection (at which time the majority of cells are considered as showing transient expression of PB transposase, EndoGalC, and the GOI), cells were harvested and subsequently treated with IB4SAP, as shown in Fig. 2,

prior to cultivation in normal medium. Cells with strong (but transient) expression of EndoGalC should survive the IB4SAP treatment and PB-mediated chromosomal integration of the GOI *via* the TTAA elements could occur concomitantly. Thus, almost all surviving colonies would express GOI together with the α-Gal epitope on their cell surface, probably due to the loss of pTransIEnd during cell growth. This system does not leave parts of pTransIEnd and the plasmid backbone included in the GOI expression vector in host genome and therefore, appears to be superior to the gene delivery system shown in Fig. 2, which may leave a genetic 'scar' such as the EndoGalC/GOI expression vectors in the host genome. Unfortunately, even with this new PB-based gene delivery system using pTransIEnd, it is still impossible to achieve targeted integration of the GOI at the desired locus.

2.2. Isolation of genome-edited non-human mammalian clones by employing targeted toxin-based drug-free selection systems

The targeted toxin technology is also useful for isolation of genome-edited mammalian cells. The most remarkable property of these genome editing systems is the acquisition of bi-allelic knockout (KO) cells (in which target gene expression is completely suppressed) directly after single transfection with DNA or mRNA, although its efficiency depends on the type of cells used, the type of genome editing systems employed, and transfection efficiency. When CRISPR/Cas9-mediated genome editing is used, co-transfection with gRNA and hCas9 expression vectors, one of which includes the drug resistance gene, is frequently employed. In this case, cells are always incubated in media containing selective drugs such as puromycin or G418 for short periods (2-4 days) 1 or 2 days after transfection to remove the untransfected cells [50-52]. During this short period, genome editing in the target locus could have occurred in the transfected cells. However, this transient selection with antibiotics often causes chromosomal integration of Cas9 or the gRNA expression unit, which may occasionally affect cell survival and function, especially when they are integrated into the loci required for cell survival. Furthermore, transient treatment with selective drugs often allows survival of some untransfected cells, which may decrease the overall efficiency of the generation of genome-edited cells. Therefore, another way to select genome-edited cells without the need to use selective drugs is required.

Recently, we found that the targeted toxin-based drug-free selection system shown in Fig. 2 is also useful for enrichment of genome-edited cells. The mechanism of this system is schematically depicted in Fig. 3. In brief, mammalian cells (except from humans and Old World monkeys) were transfected with three expression vectors, each of which carried gRNA, hCas9, or the EndoGalC gene. Once these three vectors are introduced inside a cell, EndoGalC protein expressed from the EndoGalC expression construct will digest the cell-surface α-Gal epitope, as previously shown in Fig. 2. Simultaneously, mutations at the target locus will be induced by the action of the gRNA/hCas9 complex, derived from the simultaneously introduced gRNA and hCas9 constructs. These KO cells with the loss of α-Gal epitope expression can be enriched by short-term treatment with IB4SAP to eliminate the untransfected cells and those expressing EndoGalC weakly. Thus, almost all of these surviving clones are considered to be genome-edited due to the co-expression of gRNA and hCas9. Molecular biological analysis of isolated clones revealed that 4.1 to 8.3 % and 8.3 to 58.3 % of the surviving clones were identified as bi-allelic and mono-allelic KO cells, respectively (Watanabe *et al.*, unpublished results). This system can be further modified by using all-in-vectors that confer simultaneous expression of gRNA and hCas9. For example, PEFs are first transfected with two hCas9 expressing vectors, each of which carries gRNA targeted to α-GalT gene or gRNA targeted to the GOI. After 6 days of transfection, cells are trypsinized and subjected to a short incubation with IB4SAP, prior to cultivation in normal medium. Molecular biological analysis of the emerging colonies exhibiting the loss of α-Gal epitope expression demonstrates that almost all the clones tested had indel mutations in the GOI (Sato *et al.*, unpublished results). As previously mentioned, disruption of the α-GalT gene does not affect porcine cell function. Therefore, this novel technology appears to be useful for the production of cloned domestic animals with mutations in the target loci.

2.3. Isolation of single-base genome-edited cells with no antibiotic selection

Traditional methods for isolating mutant cells with point mutations have depended on the use of selective

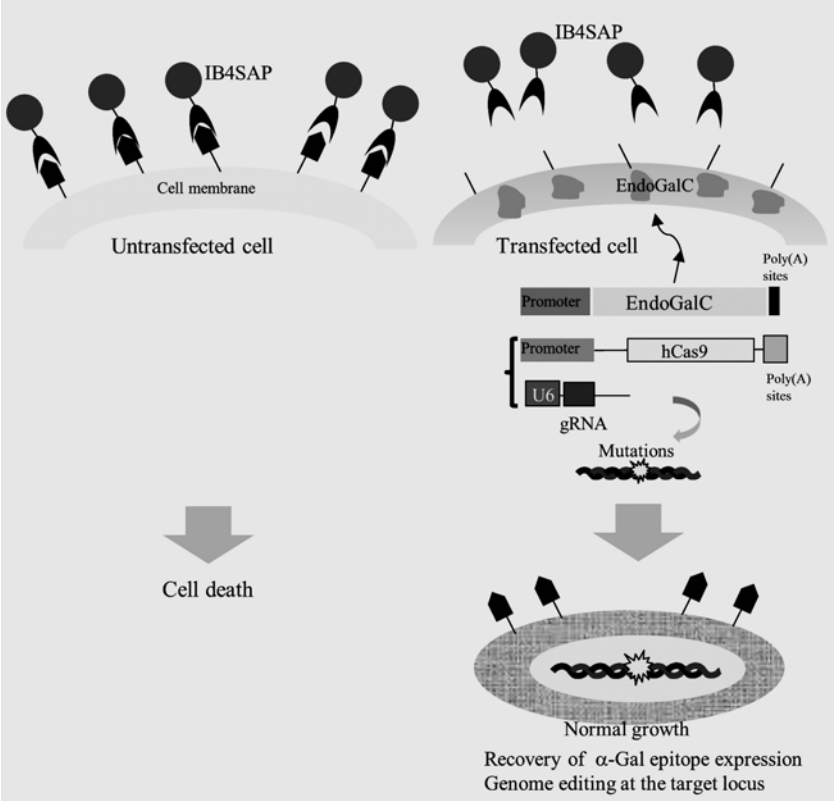

Fig. 3. Schematic diagram of a mechanism for the isolation of genome-edited cells by the targeted toxin-based selection system. Cells are transfected with three vectors, namely EndoGalC, hCas9, and gRNA expression vectors. Upon transfection, α-Gal epitope expression is ablated by the EndoGalC produced from an EndoGalC expression vector. Concomitantly, genome editing towards the target locus occurs. Three to four days after transfection, the cells are treated with IB4SAP for a short period. By this treatment, cells expressing the α-Gal epitope on their surface are eliminated. Only cells that express EndoGalC strongly and transiently can survive, and are considered to be genome-edited. U6, human U6 promoter.

drug-resistance marker genes, which always leave a genetic scar that can interfere with the study of the resulting phenotypes. The recently developed genome editing technologies have enabled precise mutagenesis in cultured cells; however, cells with a single base insertion or deletion occur at frequencies below 1% [53-55]. Therefore, it is still difficult to isolate such rare recombinant clones without the use of selective drugs. Recently, Miyaoka *et al.* [56] developed a method that allows the efficient detection of a mutation, called sib-selection (which is commonly utilized in yeast genetics to isolate a rare cell type), and the isolation of rare scarless clones with the desirable mutations, as schematically shown in Fig. 4. Cells (human iPS cells) are transfected with CRISPR/Cas9 or TALEN-related components and a 60-nucleotide (nt) single-stranded oligonucleotide (ODN) donor containing the mutation. Then, the genomic DNA isolated from the transfected cells is subjected to the TaqMan PCR (polymerase chain reaction) system coupled with the recently developed 'droplet digital PCR (ddPCR)' [57] using primer pairs and allele-specific TaqMan probes conjugated with different fluorophores that facilitate the detection of on-target homologous recombination events. If cells in a well of a 96-well plate are identified as having mutations, they are further subdivided until a rare cell type is purified. As a result, over 300-fold enrichment of the mutated cells was successfully achieved. This novel screening method for obtaining scarless genome-engineered cells will be helpful for creating precise disease models in humans or for clinical genome correction.

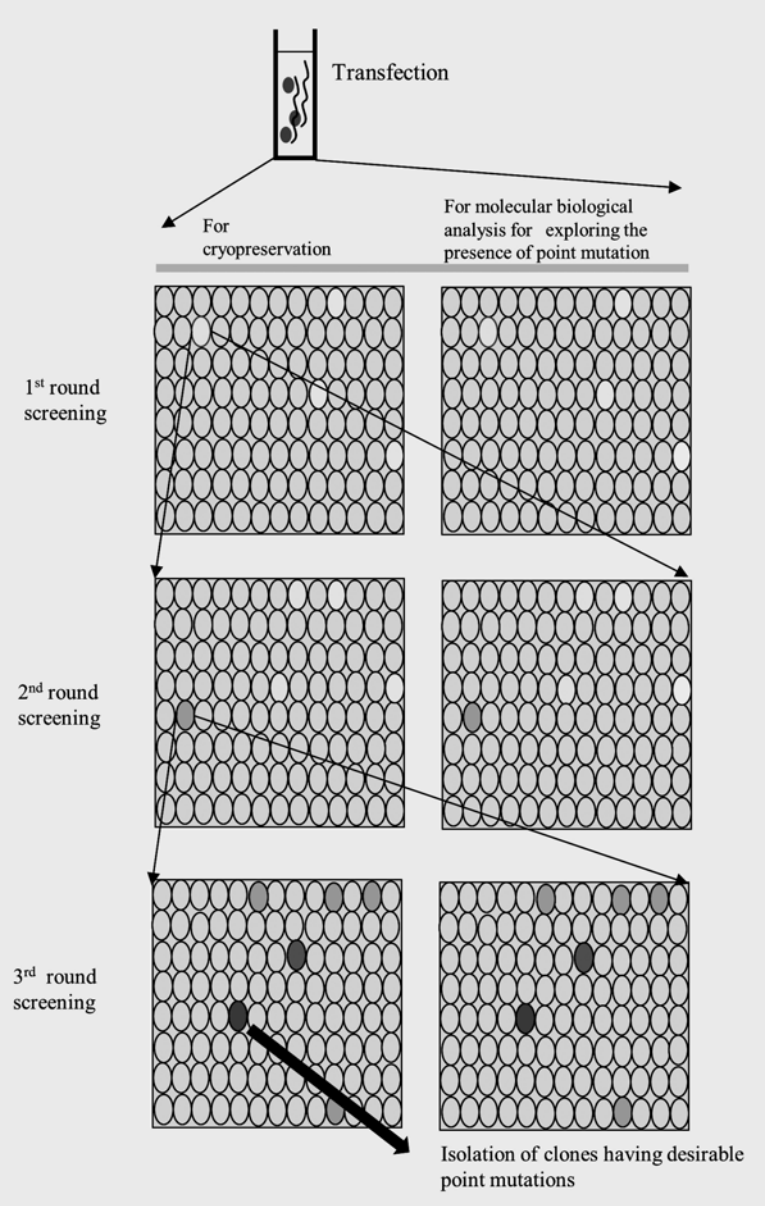

Fig. 4. Selective drug-free isolation of cells with a single base-pair mutation by using ddPCR/sib-selection. Cells are transfected with the components used for genome editing and a donor ODN, and their aliquots are then seeded into 96-well plates. In this case, the plates are duplicated: one plate is subjected to direct cryopreservation and the other is used for genomic DNA isolation. The frequency of mutations is determined by a TaqMan system coupled with ddPCR. Cells identified with higher mutant frequencies are recovered by thawing from the cryopreserved plate and are plated into a fresh 96-well plate for sib-selection. This process is repeated until the mutants are sufficiently enriched for clonal isolation.

CONCLUSION

Recent advances in genome editing technology enable us to manipulate the genome in an easier manner; however, the isolation of genome-engineered clones without the need for selective drugs in a non-expensive and non-laborious manner is still under development. Targeted integration of the GOI into a desired locus of the host genome and subsequent drug-free selection of gene-engineered clones needs to be achieved in the future.

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CONFLICT OF INTERETST STATEMENT

The authors declare no financial or commercial conflict of interest.

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