Trends in Biotechnology

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Review

Strategies for the Enrichment and Selection of Genetically Modified Cells

Chonghua Ren,[1,2,3,4] Kun Xu,[2,4] David Jay Segal,[3] and Zhiying Zhang[2,*]

Programmable artificial nucleases have transitioned over the past decade from ZFNs and TALENs to CRISPR/Cas systems, which have been ubiquitously used with great success to modify genomes. The efficiencies of knockout and knockin vary widely among distinct cell types and genomic loci and depend on the nuclease delivery and cleavage efficiencies. Moreover, genetically modified cells are almost phenotypically indistinguishable from normal counterparts, making screening and isolating positive cells rather challenging and time-consuming. To address this issue, we review several strategies for the enrichment and selection of genetically modified cells, including transfection-positive selection, nuclease-positive selection, genome-targeted positive selection, and knockin-positive selection, to provide a reference for future genome research and gene therapy studies.

Highlights

Plasmid encoded marker genes can be used for the enrichment of genetically modified cells by selecting transfection-positive cells, and surrogate reporters can be used for the enrichment of genetically modified cells by selecting nuclease-active cells.

Endogenous marker genes not only indicate genetically modified cells directly but also can be used as a cotargeting strategy to enrich cells with targeted modifications at other unlinked loci of interest.

Resection and annealing methods can enable efficient error-free targeted insertions, even in nondividing cells such as neurons.

Although knockin selection markers are ideal for selecting precise integration-positive cells, further excision of the selection marker cassette is required. Alternative and more efficient strategies for enrichment and selection of genetically modified cells should be explored.

The Need to Enrich and Select Genetically Modified Cells

The development of programmable artificial nucleases such as **zinc finger nucleases (ZFNs)** (see Glossary), **transcription activator-like effector nucleases (TALENs)**, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems have made genomic modification much more efficient. The most intense interest has transitioned from FokI-based cleavage platforms (ZFNs and TALENs) to RNA-guided systems (such as **CRISPR/Cas9** and **CRISPR/Cpf1**) over the past decade [1–7]. Generally, genomic modification can be introduced by repairing nuclease-induced **double-strand breaks (DSBs)**. Small nucleotide **insertions and deletions (indels)** can be produced as a result of the error-prone **nonhomologous end joining (NHEJ)** repair pathway [8]. **Microhomology-mediated end joining (MMEJ)**, an alternative nonhomologous end joining repair pathway, can also mediate efficient gene knockin [9]. Specific base pair changes or gene insertions can be achieved through the **homology-directed repair (HDR)** pathway, for which a homologous donor DNA must be supplied [10]. **Homology-mediated end joining (HMEJ)** and **targeted integration with linearized dsDNA donor (TILD)** CRISPR, two more recent variations on donor types, were reported to achieve knockin with higher efficiency than the MMEJ repair pathway, where TILD had the highest integration efficiency [11,12]. **Base editing** can be also achieved without DSBs by novel dCas9 or Cas9n-cytosine deaminase fusions [13].

However, due to several limiting factors, selecting genetically modified cells from millions of treated cells is still challenging and time consuming [14]. Limited delivery efficiency, especially for difficult-to-transfect cells, makes it hard to identify the modified cells among the much greater number of transfection-negative cells. The expression efficiency of the designed nuclease may vary based on different expression strategies. Furthermore, the cleavage efficiency of artificial nucleases at a specific locus is hard to predict. Compared with the

[1]School of Life Sciences, South China Normal University, Guangzhou, 510631, China
[2]College of Animal Science and Technology, Northwest A&F University, Yangling, 712100, China
[3]Genome Center and Department of Biochemistry and Molecular Medicine, University of California, Davis, CA 95616, USA
[4]These authors contributed equally to this article

*Correspondence: zhangzhy@nwsuaf.edu.cn (Z. Zhang).

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frequency of indels induced by NHEJ-mediated DSB repair, the frequency of precise gene editing generated by the donor-dependent HDR pathway is often more than 10-fold lower. The even lower efficiency of simultaneous bi-allelic editing makes it even more difficult to isolate cells with a complete disruption of a given gene without some form of screening. Even cases of successful gene editing do not usually produce a significant discernible phenotype that can be used for screening positive cells. Taken together, the low number of desired editing events and limited strategies for their selection could greatly hamper the widespread application of artificial nucleases in gene therapy and genome engineering. Thus, improved strategies for the enrichment and selection of cells with artificial nuclease-induced mutations, among large numbers of wild type cells, will greatly facilitate the use of these genome engineering tools.

In this review, we discuss several prevalent strategies for efficient enrichment and selection of genetically modified cells, including the selecting of transfection-positive cells with **plasmid markers**, nuclease-positive cells with **surrogate reporters**, genome-targeted positive cells with **endogenous selectable genes**, and knockin-positive cells with **donor markers**. These strategies can be applied to all genome editing applications, including knockout, knockin, and precise base editing. We also present principles for designing selective constructs and future perspectives on how to establish more effective and accurate strategies.

Selecting Transfection-Positive Cells

The first barrier to achieving sufficient genetic modifications in cells is the limited delivery efficiency of nuclease proteins or expression plasmids in primary or early passage nonimmortalized cultured cells, stem cells, neuronal cells, and even some cell lines. Unacceptably low delivery efficiency in these cells will directly lead to limited numbers of cells expressing the nuclease. The large proportion of nontransfected cells might further dilute the genetically modified cells when passaging. To improve the delivery efficiency, selective biomarkers such as fluorescent proteins, antibiotic-resistance genes, and cell-surface antigens are widely used to select for transfection-positive cells [15–17].

Choice of Selection Marker Genes

When a fluorescent gene such as e*GFP, mCherry, dsRed, RFP, YFP, BFP, Cerulean,* or *Venus* is used during transfection of cells with nuclease expression vectors, the transfection efficiency can be estimated by flow cytometric counting, as well as the selection of transfection-positive cells by **fluorescence-activated cell sorting (FACS)** (Figure 1) (Table 1). Fluorescent genes have been broadly used with ZFNs, TALENs, and Cas9 (Cas9 **nickase** included) [18–27]. In addition to the advantage of visualization, the use of a fluorescent gene saves both time and labor. However, its application is usually limited by the expense of flow cytometry, which may not be available in all locations. In addition, the isolated cells are easily damaged due to exposure to strong lasers and hydrostatic pressure during the sorting process.

As an alternative strategy without the need of special equipment, selection by antibiotic resistance is also widely used for the enrichment of transfection-positive cells [18,20,25,28–30] (Figure 1) (Table 1). The neomycin, zeocin, puromycin, and hygromycin resistance genes (*Neo*^{r}, *Zeo*^{r}, *Puro*^{r}, and *Hygro*^{r}) and death receptor extrinsic apoptosis CD95ΔDD are often used for antibiotic-resistance selection. Antibiotic-resistance selection does not require any special instruments or machines, but more time is needed and random plasmid integration is hard to avoid.

Beyond fluorescent and antibiotic-resistance genes, the antigen gene *H-2K*^{k} has also been used for highly efficient sorting of transfection-positive cells by **magnetic-activated cell**

Glossary

2A peptide: a 'self-cleaving' small peptide that allows for coexpression of multiple genes from a single transcript, separated by peptide cleavage.
Base editing: a genome editing technology that can convert a specific DNA base into another at a targeted locus.
Cre/LoxP: a site-specific recombinase technology used to carry out deletions, insertions, translocations, and inversions at specific sites in the genome.
CRISPR/Cas9: a DNA-editing technology adapted from a naturally occurring genome editing system in bacteria.
CRISPR/Cpf1: a DNA-editing technology analogous to the CRISPR/Cas9 system but originating from *Prevotella* and *Francisella*.
Donor markers: marker genes supplied as donors and integrated into the genome for selection.
Double-strand breaks (DSBs): one type of DNA damage that causes a break in both strands of the DNA helix.
Endogenous selectable genes: genes with specific characteristics in cells that are sensitive to corresponding treatments.
Fluorescence-activated cell sorting (FACS): a specialized type of flow cytometry used for cell counting, sorting, and biomarker detection.
Homology-directed repair (HDR): a naturally occurring nucleic acid repair system where a homologous template is supplied.
Homology-independent targeted integration (HITI): a homology-independent targeted integration repair pathway that allows for robust knockin in both dividing and nondividing cells.
Homology-mediated end joining (HMEJ): a DNA repair pathway that uses long homologous sequences to align the broken strands before joining.
Insertions and deletions (indels): a molecular biology term for an insertion or deletion of a small number of bases in a DNA sequence.
Magnetic-activated cell sorting (MACS): a cell separation method

sorting (MACS) using a magnetic bead-conjugated antibody [31,32] (Figure 1). Compared with the fluorescent strategy, MACS requires much simpler magnetic instruments and is suitable for sorting cells that are sensitive to lasers or pressure. However, one problem with this strategy is the interference of native antigens in some specific cell types.

Researchers should choose appropriate markers based on their experimental conditions and requirements. Combining different kinds of marker genes may also improve gene editing outcomes [33].

Expression Strategies for Selection Markers

There are several ways that selection markers can be incorporated into the nuclease expression system for the selection of transfection-positive cells (Figure 1). First, a separate plasmid containing the selection marker genes can be cotransfected into the cells, together with the nuclease expression vectors. Second, the marker gene can be inserted into the nuclease expression plasmid as a separate expression cassette controlled by an independent promoter, which means the expression of the marker gene and the nuclease will not affect each other. For these two strategies, the expression of the nuclease is independent of, and thus not assured by, marker gene expression. A third approach to address this concern is to fuse the marker gene to the end of the nuclease gene directly. In this approach, the nuclease and marker can be expressed as a fusion protein, expressed as one poly-protein that is cleaved into separate nuclease and marker proteins using a self-cleaving **2A peptide** (T2A or P2A), or expressed as a single bicistronic mRNA that expresses separate nuclease and marker proteins using an internal ribosome entry site (IRES) sequence [34,35]. In addition to selecting transfection-positive cells, the direct, 2A, or IRES fusion strategies further allow selection for nuclease expression-positive cells.

Selecting Nuclease-Active Cells

The strategies as described above focus on the selection of transfection-positive cells, which can in some cases select for expression of the nuclease. However, those strategies do not report whether the nuclease in the cells is active. Sometimes the cleavage activity of the artificial nuclease is relatively low in the transfected cells, in which case transfection-positive selection strategies will not be useful.

To address this issue, the concept of surrogate reporters has recently emerged, based on the hypothesis that artificial nucleases with cleavage activity on an extra-chromosomal surrogate reporter have a higher probability of cutting the endogenous genomic target [34,36,37]. The surrogate reporter shares the target sequence with the target genomic DNA and can be used not only for measuring the nuclease cleavage activity but also for the high throughput screening of nucleases with high efficiency (Figure 2A).

NHEJ-Based Surrogate Reporters

Kim and colleagues were the first group to establish a series of NHEJ-based surrogate reporters for the enrichment of genetically modified cells (Table 1) [38]. These surrogate reporter constructs were characterized by a transfection marker gene, the nuclease target sequence, and a frame-shifted reporter gene (Figure 2B). The marker gene, such as the fluorescent gene *mRFP*, was used to evaluate the transfection efficiency. Once the nuclease introduced a DSB on the target sequence within the surrogate reporter construct, small random indels could be formed through the error-prone NHEJ repair pathway, leading to the correction of the reporter genes with a 1/3 frequency. The functional reporter gene was further used for measuring the nuclease cleavage activity, as well as selecting genetically modified cells. Several NHEJ-based

based on magnetic isolation technology.
Microhomology-mediated end joining (MMEJ): a DNA repair pathway that uses 5–25 base pair microhomologous sequences to align the broken strands before joining.
Nickase: a modified version of Cas9 protein that cleaves DNA and results in single-strand breaks.
Nonhomologous end joining (NHEJ): a pathway that repairs double-strand breaks without the need for a homologous template.
PiggyBac transposon: a mobile genetic element that efficiently transposes between vectors and chromosomes via a 'cut and paste' mechanism.
Plasmid markers: selectable marker genes in a plasmid.
Precise integration into target chromosome (PITCH): a DNA repair pathway harnessed from MMEJ for gene knockin.
SSA: single strand annealing of homologous repeat sequences that flank a DSB, which causes a deletion rearrangement between the repeats.
Surrogate reporters: contain the target sequence and faithfully reflect a nuclease's activity on the chromosome in the same cell.
Targeted integration with linearized dsDNA donor (TILD): a DNA repair pathway that uses a linearized dsDNA donor with long homologous sequences to integrate.
Transcription activator-like effector nucleases (TALENs): artificial restriction enzymes generated by fusing a transcription activator-like effector DNA-binding domain to a DNA-cleavage domain, which are functional as dimers.
Zinc finger nucleases (ZFNs): artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain, which are functional as dimers.

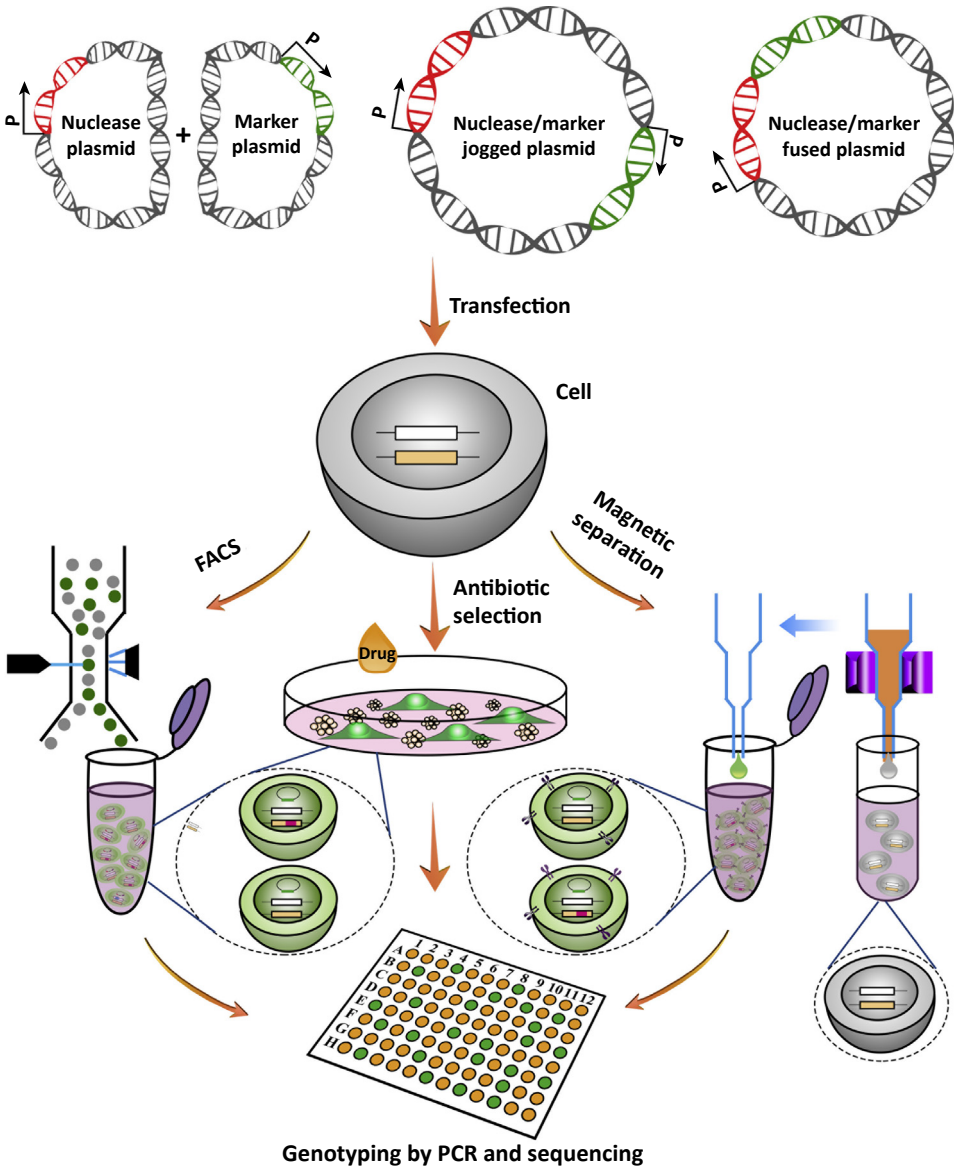

Figure 1. Strategies for the Selection of Transfection-Positive Cells. Three kinds of marker plasmids (nuclease/marker separated plasmids, nuclease/marker jogged plasmids, and nuclease/marker fused plasmids) shown at the top of the figure can be cotransfected with artificial nucleases into cells for selection. Transfection-positive cells with higher proportions of genetic modifications are selected by fluorescence-activated cell sorting (FACS), antibiotic selection, or magnetic separation and plated into multiple-well cell culture dishes. Genetically modified single colonies can grow for genotype detection by PCR and Sanger sequencing.

surrogate reporters applying different reporter genes, including *eGFP, H-2K*^{k}, and *Hygro*^{r}, have been developed for enriching genetically modified cells by flow cytometric sorting, magnetic separation, and antibiotic-resistant selection [33,38–44]. The traffic-light reporter vector also can be used as a surrogate reporter to enrich genetic modified cells based on the NHEJ DNA repair pathway [34]. Another NHEJ-based surrogate reporter using blasticidin S resistance gene (*Bs*^{r}) has been also reported recently [45].

Table 1. Plasmids Used for Selection and Enrichment

Enrichment strategies		Vector names	Transfection-selective genes	Nuclease-active reporter genes	Addgene ID	Refs
Transfection-positive selection	Separate marker plasmid	phGFP-S165T	*GFP*	–	–	[15]
		pBABE-puro	*Puro^r*	–	#1764	[112]
		pMACS K^k	*H-2K^k*	–	–	[31]
	Nuclease/marker jogged plasmid	pCas-Guide-EF1a-GFP	*GFP*	–	–	[113]
		pAAV-U6-sgRNA-CMV-GFP	*GFP*	–	#85451	[114]
	Nuclease/marker fused plasmid	pSpCas9(BB)-2A-GFP	*GFP*	–	#48138	[20]
		pSpCas9(BB)-2A-Puro V2.0	*Puro^r*	–	#62988	[20]
		pSpCas9n(BB)-2A-GFP	*GFP*	–	#48140	[20]
		pSpCas9n(BB)-2A-Puro V2.0	*Puro^r*	–	#62987	[20]
		pCMV-Cas9-GFP	*GFP*	–	–	[21]
Nuclease-active selection	NHEJ-based surrogate reporters	pRGS series	*mRFP*	*eGFP*	–	
				eGFP-eGFP	–	[38]
				Hygro^r	–	[41]
				H-2K^k	–	
		Traffic-light reporters	*Puro^r*	*mCherry*	#31482	[34]
			BFP		#31481	
		pBSR, pBS SK mCherryROSAegfp	*mCherry*	*Bs^r*	#66950	[45]
				eGFP	#54322	
		pNHEJ-RPG	*dsRed*	*Puro^r, eGFP*	#85931	[14]
	SSA-based surrogate reporters	Traffic-light reporters	*Puro^r*	*mCherry*	#31482	[34]
			BFP		#31481	
		pRep.eGFP	*dsRed*	*eGFP*	–	[53]
		pSSA-RPG	*dsRed*	*Puro^r, eGFP*	#85932	[14]
		C-Check	*eGFP*	*AsRed*	#66817	[37]

The main limitation of the NHEJ-based surrogate reporter is its relatively low sensitivity, since in theory only 1/3 of the repair products can restore the reporter gene to functionality. To overcome this disadvantage, Kim and colleagues developed a modified surrogate reporter with double frame-shifted e*GFP* genes (Table 1), which improved the probability of *eGFP* frame correction to 2/3 after NHEJ-mediated repair [41].

Single-Strand Annealing (SSA)-Based Surrogate Reporters

In addition to the NHEJ-based surrogate reporters, the growing popularity of **SSA**-based reporters has been described in past years (Table 1). These reporters are characterized by an interrupted nonfunctional reporter gene with direct repeats (DRs) flanking the nuclease target sequence. Once the nuclease cleaves its target, the DSBs will be repaired through the SSA pathway, deleting the region between the DRs and leading to the precise restoration of the selective reporter gene [46,47]. Fluorescent, antigen, and antibiotic-resistance genes have all been applied for the SSA-based surrogate reporters [34,48–50].

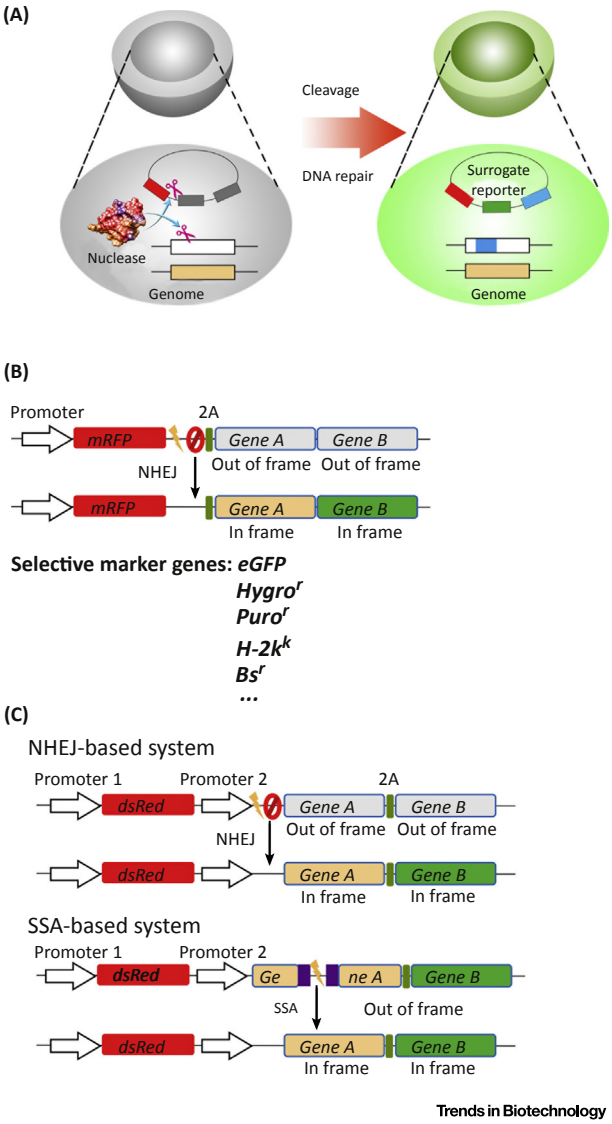

Figure 2. Schematic Drawing of the Enrichment Principle with Surrogate Reporters. (A) Principle of surrogate reporters for enrichment. (B) Nonhomologous end joining (NHEJ)-based single open reading frame (ORF) surrogate reporter series established by Kim and colleagues. Several selective marker genes are listed below for reference. (C) Dual ORF surrogate reporters, including NHEJ- and single-strand annealing (SSA)-based systems, which repair the double-strand breaks and make the maker genes in frame using the NHEJ and SSA pathways separately.

SSA-based surrogate reporters (Figure 2C) can be used not only for nuclease activity detection, but also to select genetically modified cells. For example, goat mammary epithelial cells and sheep fetus fibroblast cells modified by ZFNs were enriched using SSA-based Puro^r and mRFP-eGFP surrogate reporters [51,52]. A similar surrogate reporter, described as the 'C-Check' system, has been reported for FACS sorting and enrichment of genetically modified cells [37]. An optimized SSA-RPG surrogate reporter system, which combined the *dsRed* marker gene and the *Puro^r-eGFP* selective reporter genes (RPG) in a single plasmid vector has also been established. The *dsRed* marker gene was cloned within an individual expression

cassette, while the *Puro*^{r} and *eGFP* genes were joined by a T2A element as a dual reporter that would facilitate the selection of genetically modified cells by either puromycin-resistant selection or FACS [14]. The SSA-RPG surrogate reporter system has been successfully employed for the modification of human HEK293, mice C2C12, chicken DF-1, porcine pK-15, and primary fibroblast cells with high efficiency [53–56].

Comparison of NHEJ- and SSA-Based Surrogate Reporters

At present, most surrogate reporter systems function based on NHEJ or SSA DSB repair pathways. One study found that SSA-RPG surrogate reporters with 200 bp DR lengths were much more sensitive for detecting nuclease activity than similarly constructed NHEJ-based RPG reporters. An SSA-based reporter was able to identify more nuclease-positive cells due to its higher sensitivity [57], and it significantly improved the possibility of obtaining the desired genetically modified cell clones [14]. Nevertheless, the SSA-RPG and NHEJ-RPG surrogate reporters share similar enrichment efficiencies for genetically modified cells (up to 85% mutation efficiency and 34.8-fold improvement compared with unenriched groups), indicating that the genomic modification within selected positive cells may be independent of the repair pathways in surrogate reporters. He and colleagues also found that both NHEJ (or RGS) and SSA systems were effective for enriching edited porcine cells; however, the RGS reporter provided better enrichment than the SSA reporter in their study [58]. Since different reporter constructs and different cell types were used between these two studies, further systematic research may be needed to address this issue.

Even though there is some difference between NHEJ- and SSA-based surrogate reporters, both of them can be applied to FokI-based cleavage platforms (ZFNs and TALENs) and to RNA-guided CRISPR systems. Similarly, new HDR-based surrogate reporters also can be efficiently used for enrichment, especially for HDR-mediated knockin enrichment when compared with NHEJ- and SSA-based systems. Compared with the strategy of selecting transfection-positive cells, the surrogate reporter system can select the nuclease-active cells further on the basis of transfection-positive cells, which means a higher enrichment efficiency can be achieved for surrogate reporter systems.

Selection Using Endogenous Genes as Markers

Direct Selection by Targeting Genes with Specific Characteristics

The most efficient strategy for the selection of genetically modified cells is direct selection by targeting endogenous genes with specific characteristics. The common approach to this selection strategy is to use a compound or drug that is toxic to wild type cells due to the expression of a particular endogenous gene, while the targeted mutant cells will survive since they lack the ability to form toxic compounds (Figure 3A).

In yeast, α-aminoadipate can be used for LYS2^{-} and LYS5^{-} mutant cell selection [59], methyl mercury for MET2^{-} and MET5^{-} mutant selection [60], and both ureidosuccinate and 5-fluoroorotic acid for URA3^{-} cells [61–63]. The negative selective marker gene *CAN1* has also been used to monitor the mutation frequency at the targeting locus. Nonsense mutations induced by CRISPR/Cas9 in the *CAN1* gene can be selected with media containing canavanine (a toxic arginine analogue) [64,65]. Mutations created by artificial nucleases on these target genes can be selected by plating the yeast transformants on medium supplemented with the appropriate inhibitors.

In mammalian cells, mutations in the dihydrofolate reductase (DHFR) gene could be isolated in Chinese hamster ovary cells after *DHFR* mutagenesis and exposure to high-specific activity [^{3}H]

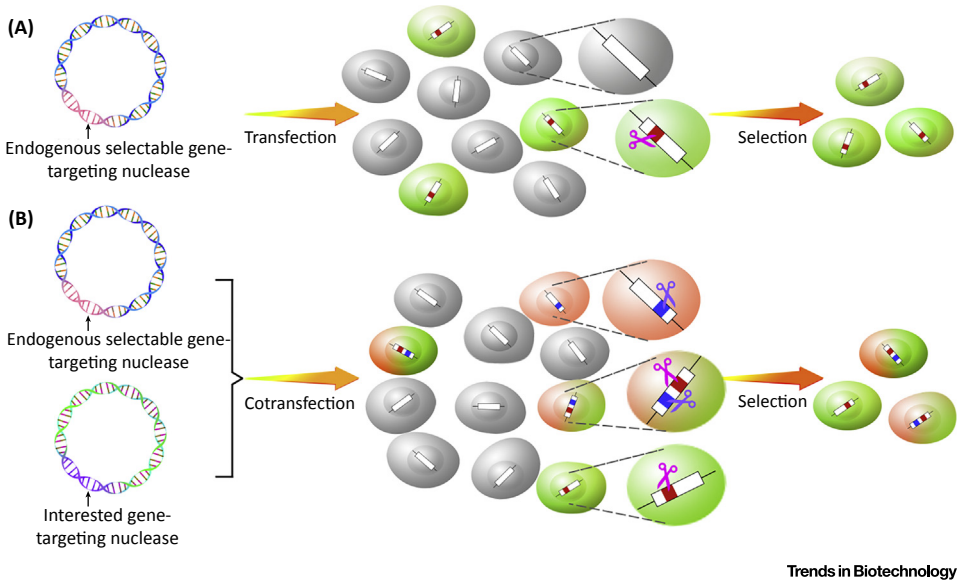

Figure 3. Enriching Genetically Modified Cells by Editing Endogenous Selectable Genes. (A) Selection of genetically modified cells directly by targeting endogenous selectable genes. (B) Enrichment of genetically edited cells by cotargeting another endogenous selectable gene in the same cell.

deoxyuridine as a selective agent [66]. Targeting the *ATP1A1* gene, which encodes the Na^+K^+-ATPase that is responsible for maintaining the electrochemical gradients of Na^+ and K^+ across the plasma membrane of animal cells, can be selected by incubation with ouabain, a highly potent plant-derived inhibitor of the Na^+K^+-ATPase [67]. In livestock, cells surviving after IB4SAP treatment would be negative for α-Gal epitope expression, which in turn indicates the generation of *GGTA1* bi-allelic knockout cells [68]. Similarly, host genes essential for the toxic effects of anthrax and diphtheria toxins have been identified using a screening system-based gene function and high-throughput sequencing that enabled cells with targeted gene mutations to survive after inhibitor or toxin treatment [69].

Enrichment by Cotargeting Endogenous Selectable Genes

The special genes mentioned above can be used not only for selecting cells with mutations at those loci, but also for enriching cells with targeted modifications at other unlinked loci of interest. Compared with the episomal plasmid surrogate reporters, using endogenous selectable genes can make the selection of genetically modified cells depend on events at chromosomal loci. Conceptually distinct and elegant genetic approaches based on the creation of 'classical' gain-of-function alleles was first developed in *Caenorhabditis elegans* [70,71]. The 'coconversion' strategy increased the odds of detecting phenotypically indistinguishable gene mutation through the simultaneous coconversion of a mutation in an unrelated target that causes a visible phenotype (Figure 3B). Distinct from the surrogate reporters described above, double editing events on two different endogenous loci within a single cell, induced by separate nucleases, are independent. However, different guide RNAs (gRNAs) share the same Cas9 nuclease, and the occurrence of a genome editing event on one locus should enhance the probability of targeting on another locus in the same cell after selection. A related approach has been described to isolate human cells harboring NHEJ-driven mutations by cotargeting the *HPRT1* gene [29]. A more recent study found that coincident insertion of a drug-selectable marker at one control site enables high-efficiency genome knockin at an unlinked targeted site in mouse embryonic stem cells [72]. In addition, ouabain has been used for a marker-free

coselection strategy for CRISPR-driven NHEJ- and HDR-based editing events in human cells by cotargeting the *ATP1A1* loci and interesting genes [73].

The genes *LYS*, *MET*, and *URA3*, mentioned above in yeast, are also suitable for the cotargeting concept for enriching unlinked loci of interest. The endogenous marker gene enrichment strategy is different from the marker plasmid or surrogate reporter system. This strategy is independent of exogenous markers and can monitor modifications that occur in the genome that do allow for selecting enriched cells to generate individual clones for analysis. Some drawbacks of this strategy are the potential to generate chromosomal translocations and other unpredictable rearrangements.

Selection Using Knockin Selective Donor Markers

Although the three strategies mentioned above can be used for knockin enrichment, they are still better suited to enrich knockout cells, and reports of their use for knockin are uncommon. Knockin of a selective reporter construct is the most common strategy for selecting genetically modified cells, but an additional step of excising the integrated selection markers is often necessary.

Integration Pathways for Selective Marker Knockin

In order to achieve high efficiency for both knockout and knockin gene modifications, a donor plasmid with a selective marker gene is a useful option. When the DSBs are induced by a designed artificial nuclease, the selective marker gene (as well as other genes of interest, if necessary) can be integrated into the target genomic site through a DNA repair pathway that will depend on the donor design. The selective marker gene, once integrated, will allow the efficient selection of the knockin-positive cell clones. Five main strategies, NHEJ, MMEJ, HDR, HMEJ, and TILD, have been reported to mediate the targeted integration of donor DNA (Box 1) [74]. Conceptually analogous to the strategies previously described to select cells harboring NHEJ-mediated gene-disruption events, a HDR-based primary editing event to incorporate a

Box 1. Integration Pathways for Donor Marker Genes

The NHEJ-based **homology-independent targeted integration (HITI)** strategy takes advantage of the blunt-end cleavage by Cas9 to insert a blunt-end linear donor DNA at the cleavage site. HITI has been successfully utilized in both dividing and nondividing cells (Figure IA) [102]. HITI insertions can occur in either orientation but are expected to occur more frequently in the forward than the reverse direction, since an intact gRNA target sequence remains in the reverse. Alternatively, **precise integration into target chromosome (PITCH)** can be achieved with high efficiency by the MMEJ pathway, which only requires extremely short microhomologies ($\leq$40 bp) to the donor construct (Figure IB) [77]. The PITCH donor construct can be easily generated by adding the microhomologies by one-step PCR, and a very nice web tool for designing the pitch donor, PITCh designer 2.0, is available [103]. However, both HITI and MMEJ may cause indels at junctions, similar to NHEJ-based integration [104].

HDR, which requires donor DNA flanking with long homology arms, usually 0.5 to 2 kb, is the most commonly used pathway for knockin manipulation. HDR-mediated knockin selection is appropriate for both large fragment deletion and exogenous gene overexpression (Figure IC) [105]. Circular plasmids, linearized plasmids, and PCR fragments harboring selective marker genes and appropriate homology arms have been used for precise knockin integration and positive selection [37,76,106–110]. Circular plasmids can also be linearized or double-cut inside the cell by designed nucleases, which improves the recombination efficiency compared with using circular plasmids [84,111]. Partially inspired by these observations, the newly described HMEJ is a knockin strategy using a donor DNA with long homology arms, which is designed to be cut out of its plasmid by the same Cas9 nuclease that is employed to cleave the intended integration site on the chromosomal DNA (Figure ID) [11]. HMEJ was reported to have a higher knockin efficiency than other existing strategies in many systems, including cultured cells, animal embryos, and tissues *in vivo* [11], possibly because the donor becomes a linear fragment at approximately the same time the targeted DSB is made on the chromosome. Compared with HMEJ, for which additional cleavage of the donor plasmid is needed, TILD-CRISPR uses a linearized

donor fragment and achieved much higher knockin efficiency than other targeting strategies in mouse embryos, as well as brain tissue (Figure IE) [12].

In conclusion, a primary limitation of HDR-based strategies is that the enzymes supporting these events are usually only active during the S and G2 phases of the cell cycle, restricting the efficient use of HDR to only dividing cells. NHEJ- and MMEJ-based integration methods, such as HITI and PITCH, can enable cell-cycle-independent targeted insertions, but often in association with indels at the joint sites. Resection and annealing methods, such as HMEJ and TILD, are cell-cycle-independent but avoid error-prone junction repair, allowing HDR-like precision of targeted integration even in quiescent cells.

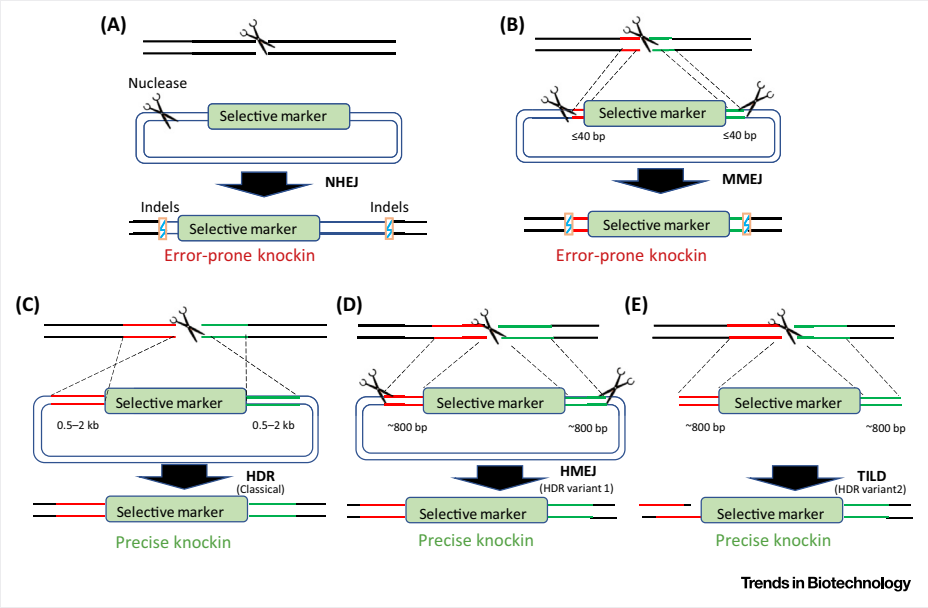

Figure I. Marker Gene Knockin Strategies to Select the Genetically Modified Cells. Five pathways, namely (A) nonhomologous end joining (NHEJ), (B) microhomology-mediated end joining (MMEJ), (C) homology-directed repair (HDR), (D) homology-mediated end joining (HMEJ), and (E) targeted integration with linearized dsDNA donor (TILD), are used for the integration of donor marker genes.

selectable marker at one locus could be accompanied by one or more independent user-specified HDR-mediated edits at other loci, since multiple independent HDR events could, in theory, occur simultaneously [75].

Selective Marker Cassettes for Knockin Donors

The selective marker genes used in the donor construct can be flexible. The choice of fluorescent genes or antibiotic-resistance genes depend on the circumstances of the modification. All of the marker genes mentioned so far are suitable, and the combination of different marker genes makes the selection of mono-allelic knockins more efficient [76,77].

In addition to mono-allelic integration, highly efficient bi-allelic targeting has been achieved using FACS-based dual-fluorescent selection strategies [78]. The presence of both fluorescent markers within the same cell clone indicates successful integration at both alleles. A different novel and efficient approach for screening bi-allelic gene-edited cells combined the surrogate reporters and the fluorescent-antibiotic selection strategy together. Two different SSA-based

surrogate reporter cassettes (Puro^r-eGFP and Zeo^r-mRFP) were incorporated into a pair of donor plasmids, generating the surrogate reporter-integrated donor (Rep/Don) constructs used for both the surrogate reporters for measuring the nuclease activity and the knockin donors for screening bi-allelic targeted cell clones [54].

The strength of the promoter driving the selective marker genes within the knockin donor constructs is an important consideration, since some promoters may not work well or might be silenced by DNA methylation or other modifications [78]. An alternative strategy is to integrate a selective marker gene without a promoter, the expression of which would be controlled by the upstream promoter of the endogenous target gene. However, the risk of this strategy is that many endogenous promoters are not strong enough to drive the expression of marker genes for efficient selection, so only genes that are actively transcribed can be selected using this approach [74].

Strategies for HDR Efficiency Enhancement

Traditional homologous recombination can be used to generate knockins, but its low efficiency remains problematic. NHEJ and HDR are the two main DNA repair pathways when donor DNA is available and they compete with each other after DNA breaks, but HDR has a much lower efficiency compared with NHEJ; therefore suppressing NHEJ and promoting HDR become important goals.

To inhibit NHEJ activity, the chemical substance Scr7 that targets DNA ligase IV, a key enzyme in the NHEJ pathway, increases the efficiency of precise genome editing [79]. A gene silencing method to suppress DNA ligase IV and KU70 protein, another key component associated with the NHEJ pathway, also improved the efficiency of HDR [79]. Another method to suppress the function of DNA ligase IV used adenovirus 4 (Ad4) E1B55K and E4orf6 proteins, which mediate the ubiquitination and proteasomal degradation of DNA ligase IV, coexpressed with Cas9 nuclease to improve the efficiency of HDR up to eight fold in both human and mouse cell lines [80]. The chemical RS-1 also increased knockin efficiency several fold [81]. Direct coexpression of HDR-related protein Rad52 also demonstrated enhanced effects on HDR [56]. *p53* inhibition is another new strategy to increase the frequency of HDR, as it would permit editing cells in the S phase of the cell cycle, which is preferred for HDR repair [82]. Apart from manipulating DNA repair proteins, the types of donor DNA also have a big impact on HDR efficiency. Circular plasmid, circular plasmid linearized *in vivo* with a nuclease, and prelinearized double-strand DNA will integrate their marker genes with different efficiency (see Figure IC–E in Box 1) [11,12]. The concentration of the repair DNA template and the length of the homology arms in donor DNA also has a large effect on HDR efficiency [83,84]. Furthermore, for single-stranded oligodeoxynucleotide (ssODN) donors, the HDR efficiency depends on the length and symmetric or asymmetric properties of homology arms [85]. We will not extensively describe ssODN donors here because this type of donor is not appropriate for independent marker gene knockin. To achieve the highest HDR efficiency, a combination of two or all three strategies mentioned above is highly recommended.

Excision Strategies for Integrated Selection Markers

The excision of selection marker genes from the chromosome is another key consideration, since their presence may affect cell behaviors, especially in the context of precise gene editing for therapy. A common strategy is to perform a first round of integration using a positive selective gene for the knockin cell clones, followed by a second round of excision using a negative selection marker gene like thymine kinase (*tk*). Using donors with such genes

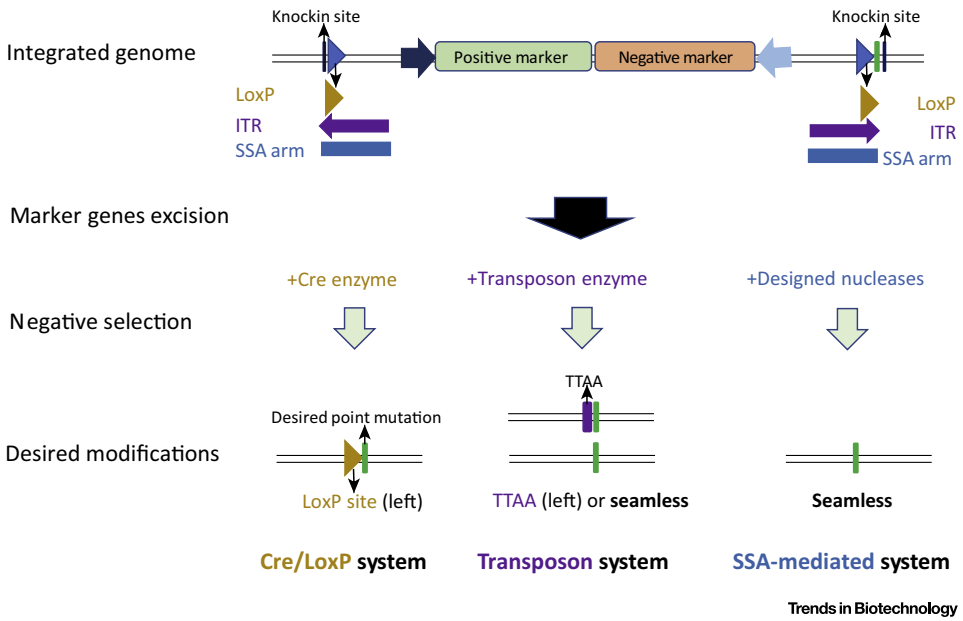

Figure 4. Marker Gene Wipe-out Strategies for Precise Genome Editing and Strategies for Marker Gene Excision. Cre/LoxP (left, gold), transposon (middle, purple), and single-strand annealing (SSA) (right, blue) methods are included. ITR, inverted terminal repeat.

incorporated, cells can survive the final selection only when the selection cassettes have been successfully removed from the chromosome.

As shown in Figure 4, **Cre/LoxP** is the classical system used for the excision of selection cassettes. The fragment flanked by two LoxP sites can be excised by the Cre recombinase, leaving a single LoxP site as a 'scar' behind [86–89]. A **PiggyBac transposon** system was used to excise the selection cassettes with a TTAA scar left behind and developed to excise from endogenous TTAA sites, leaving no scar behind [90–92]. Kuhn and Chu reported another pop-in/pop-out strategy for 'seamless' genome editing, which utilizes CRISPR/Cas9-mediated

Table 2. Comparison of Selected Enrichment Strategies

Enrichment strategies	Components needed	Difficulty in preparation	Applicable modification types	Enrichment efficiency	Drawbacks
Transfection-positive selection	Separate or fused marker genes	+	**Knockout**/Knockin	★	Random integration of marker genes is possible; drug selection and FACS sorting may be harmful for cells.
Nuclease-active selection	Surrogate reporters	+	**Knockout**/Knockin	★★	Cleaved surrogate reporter plasmid may be integrated into the genomic DSB site; drug selection and FACS sorting maybe harmful for cells.
Endogenous markers	Endogenous gene targeting nucleases	++	**Knockout**/Knockin	★★★	Unintended artifacts might be created, including chromosomal translocations and unpredictable outcomes when endogenous marker genes are disrupted.
Knockin enrichment	Selectable donor fragment	+++	**Knockin**	★★★★	Another step of excising selective markers is necessary.

HDR twice for the selection cassette knockin and knockout [93]. Combining CRISPR/Cas9-mediated HDR and SSA for the knockin and excision manipulations will be a novel 'seamless' genome editing strategy.

Donor marker is the first choice for knockin integration modifications and still a good option for precise gene editing, even though another excision step is needed to remove the markers. Additionally, compared with the other three methods mentioned above, the donor marker strategy has its own advantage in bi-allelic modifications because different markers can be used conjunctively.

Concluding Remarks and Future Perspectives

Programmable artificial nucleases have emerged as a revolutionary genome engineering technology for research and therapeutic purposes. However, the difficulty of isolating genetically modified cells from the large numbers of wild type cells limits the more widespread application of these methods. In this review, we systemically summarized current enrichment and selection strategies for cells with genetic modifications induced by artificial nucleases.

Cells can be enriched by cotransfecting plasmid marker genes for selecting transfection-positive or nuclease expression-positive cells. Surrogate reporters selecting nuclease activity-positive cells based on different repair pathways are suitable for the enrichment of both knockout and knockin cases, as well as point editing and base correction. However, they enrich but do not specifically select for cells with gene edits at endogenous loci (see Outstanding Questions). Another limitation for any plasmid or naked DNA-based method is that many primary cells will not tolerate their transfection, so DNA-based reporter systems are often not useful for these applications. Specific endogenous genes sensitive to biochemicals can contribute to the selection of genetically modified cells, and the coconversion strategy can increase the odds of detecting phenotypically indistinguishable gene mutations through targeting an unrelated gene that causes a selectable phenotype. Knockin of selective markers is ideal for selecting precise integration-positive cells, but further excision of the selection cassette would be necessary for point editing and scarless genome correction. Each strategy has its own pros and cons (Table 2), which researchers should consider based on the particular experimental situation and requirements.

There have been many significant complementary advances in gene editing, in addition to the progress in enrichment and selection strategies for genetically modified cells described here. For example, it is beyond the scope of this review to discuss the significant efforts to improve on-target activity, reduce off-target activity, boost nuclease expression efficiency, and expand potential methods for nuclease delivery into cells and whole organisms, which have been well reviewed by others [74,94]. However, beyond even recent advances in selection strategies, there remains a need for even more sensitive and direct selection methods. No current method can sense indel-modified cells directly, let alone selectively eliminate mutant cells containing undesired NHEJ-mediated indels or HDR-mediated base edits. One possible approach toward this goal would be to use promoters for DNA damage response genes to promote the expression of marker genes, such as *gadd* [95–97], *p21* [98], and *E2F7* [99] in mammalian cells, and *RNR2* and *RNR3* in yeast [100,101]. Mutant cells with NHEJ-mediated indels could be labeled by the DNA damage inducible markers, which would remove or greatly reduce the obstacles for the selection of positive cells. There are still many opportunities for additional efforts and contributions to make genome engineering for both research and therapeutic purposes more accessible.

Outstanding Questions

How can the efficiency of current enrichment and selection methods be further improved? Are there more efficient strategies for the enrichment and selection of genetically modified cells?

Are there other safe endogenous marker genes that can be used as cotargets for the selection and enrichment of genetically modified cells?

Are there more efficient strategies for the seamless removal of reporter cassettes following selection for targeted integration into genomes?

How can successfully genetically modified cells be marked and identified directly? How can cells containing undesired modifications be selectively eliminated?

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Disclaimer Statement

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