Molecular Therapy
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Review

Recent advances in CRISPR-Cas9-based genome insertion technologies

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Programmable genome insertion (or knock-in) is vital for both fundamental and translational research. The continuously expanding number of CRISPR-based genome insertion strategies demonstrates the ongoing development in this field. Common methods for site-specific genome insertion rely on cellular double-strand breaks repair pathways, such as homology-directed repair, non-homologous end-joining, and microhomology-mediated end joining. Recent advancements have further expanded the toolbox of programmable genome insertion techniques, including prime editing, integrase coupled with programmable nuclease, and CRISPR-associated transposon. These tools possess their own capabilities and limitations, promoting tremendous efforts to enhance editing efficiency, broaden targeting scope and improve editing specificity. In this review, we first summarize recent advances in programmable genome insertion techniques. We then elaborate on the cons and pros of each technique to assist researchers in making informed choices when using these tools. Finally, we identify opportunities for future improvements and applications in basic research and therapeutics.

INTRODUCTION

The precise insertion of customized DNA sequences into the target genome plays a critical role in synthetic biology, genetic, and cell engineering applications, such as generating stable cell lines for producing recombinant proteins or creating transgenic mice. Translational research using genome insertion technologies for treating monogenic genetic disorders and achieving site-specific integration of chimeric antigen receptor (CAR) into T cells, natural killer cells, and other immune cells is rapidly advancing with numerous clinical trials underway.[1–5] The approaches for introducing foreign DNA into the target genome can be categorized into two main groups: random and site-specific genome insertion. Although random genome insertion by viral vectors or transposases has been extensively utilized, it often comes with risk of disrupting endogenous genes or regulatory elements.[6] In contrast, targeted genome insertion would otherwise avoid undesired outcomes and guarantee the normal function of foreign DNA.

The development and advancement of site-specific genome insertion technologies heavily rely on programmable nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR-Cas9 system, which can introduce double-strand breaks (DSBs) at desired genomic loci.[7] In eukaryotes, DSBs can trigger several mutually exclusive or complementary DNA repair pathways, including homology-directed repair (HDR), non-homologous end-joining (NHEJ), and microhomology-mediated end joining (MMEJ). All these pathways have been successfully harnessed for targeted genome insertion.[8] Recently, alternative site-specific genome insertion technologies that do not generate DSBs have also been developed.[9–14] These methods are exemplified by (1) prime editing and paired prime editing; (2) integrase coupled with programmable nuclease; and (3) transposase and CRISPR-associated transposon (CAST).

This review focuses on recent advances in technologies for site-specific genome insertion (Tables 1 and S1). We summarize efforts to improve both editing efficiency and product purity of these technologies, highlight their capabilities and limitations, discuss their applications in biology research and translational medicine, and conclude with future directions to overcome existing bottlenecks.

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DSB repair-mediated genome insertion

In eukaryotes, programmable nucleases-induced DSBs can trigger several DNA repair pathways, including NHEJ, HDR, and MMEJ, where different DNA repair mechanisms may occur simultaneously or competitively.[8] NHEJ, also known as canonical NHEJ (c-NHEJ), is the major DNA repair pathway that operates throughout the cell cycle.[15] The entire process of NHEJ involves three steps: (1) recognition and binding of the break, (2) processing of the ends, and (3) ligation of blunt ends. NHEJ is cooperatively carried out by several proteins or complexes, including Ku70, Ku80, Artemis: DNA-dependent protein kinase (DNA-PK) complex, and ligase IV.[16] As an error-prone repair pathway, NHEJ often generates a high frequency of insertions or deletions (indels) at the repaired junctions.[17] In addition to c-NHEJ, there exists another form called alternative NHEJ, also known as MMEJ. MMEJ functions during G1/early S phase, and utilizes microhomology sequences with lengths ranging from 5 to 25 bp for DSB repair. MMEJ is initiated through cooperation among various proteins including MRN (Mre11-Rad50–Nbs1)/BRCA1/CtIP complex, XRCC1 and Ligase I or III.[17] MMEJ can result in both small insertions and deletions depending on the locations of microhomologies on single-stranded DNA (ssDNA) and strategies used for MMEJ.[18] HDR represents the second major repair mechanism active during S/G2 phase in cell cycle, which occurs primarily via the synthesis-dependent strand-annealing pathway.[15] HDR involves four steps: (1) recognition and resection of DNA ends, (2) homologous pairing and formation of D loop, (3) DNA heteroduplex extension, branch migration and D loop disassociation, and (4) annealing of the extended end to complementary ssDNA, DNA synthesis, and ligation.[15,19,20] Several proteins participate in the process of HDR, including MRN complex, Rad51, Rad52, BRCA2, and RPA.[15] HDR enables precise targeted insertions and substitutions using a homology template. Taking advantages of these DSB repair pathways, various site-specific genome insertion techniques have been developed (Figure 1).

HDR-mediated knock-in

HDR-mediated knock-in is the most commonly used method for targeted genome insertion (Figure 1A). Extensive efforts have been made to enhance the efficiency of HDR-mediated genome insertion (reviewed extensively elsewhere),[21–23] which can be categorized into the following four strategies.

(1) Modulation of the nature of DSB repair systems.[24–27] Several groups have demonstrated that reducing or inhibiting core proteins involved in NHEJ, such as Ku70, Ku80, DNA-PK, and ligase IV, can increase the efficiency of HDR-mediated knock-in by 2- to 9-fold in various cell types and mice.[24–26] Conversely, overexpression or stimulation of genes involved in HDR offers another strategy to improve HDR-mediated knock-in efficiency.[28–30] For instance, it has been shown that RAD51 overexpression or addition of RAD51-stimulatory compound 1 in cell culture media can enhance HDR-mediated knock-in by up to 6-fold.[28,31] Additionally, selective inhibition of DNA-PK using AZD7648 significantly increases HDR efficiency up to 50-fold, while decreasing local indels simultaneously.[32,33] Inhibition of both DNA-PK and DNA polymerase theta, known as the 2iHDR approach, further improves integration efficiency and precision.[32] Fusion of the CtIP, a protein participating in HDR, with Cas9 has been also reported to enhance HDR-mediated knock-in efficiency by 2-fold in HEK293T cells.[27] More recently, Zuris and coworkers proposed SeLection by Essential-gene Exon Knock-in (SLEEK), a method that involves the insertion of a cargo template within an exon of an essential gene.[34] By subjecting cells with non-productive insertions and deletions to negative selection, SLEEK can achieve knock-in efficiency of more than 90% in multiple cell types. In mammalian cells, DSB-mediated p53 activation can lead to severe cytotoxicity and cell-cycle arrest.[35–37] Suzuki and colleagues demonstrated that adding cytosine stretches to the 5′-end of conventional sgRNAs substantially reduces p53 activation and cytotoxicity in human induced pluripotent stem cells (iPSCs), thus enhancing HDR without compromising bi-allelic editing.[38] Additionally, the addition of GSE56, a dominant-negative p53 mutant protein, can transiently inhibits the p53 response and enhance knock-in efficiency.[39] Apart from DSBs, several groups have utilized paired nicking strategy for efficient HDR.[40–44] For instance, the "spacer-nick" approach combining $Cas9^{D10A}$ nickase with a pair of PAM-out sgRNAs at a distance of 200–350 bp achieved up to approximately 50% knock-in efficiency in hematopoietic stem and progenitor cells (HSPCs) with minimal on-target indels.[40]

(2) Synchronization of the cell cycle to S/G2 phases.[45–48] Since HDR is limited to the S/G2 phases, arrest of cell cycles in S/G2 phases by chemical compounds (e.g., nacodazole and aphidicolin) have proven effective to enhancing HDR-mediated knock-in efficiency.[45–48] For example, the utilization of nacodazole to arrest cells in G2/M phases increased HDR-mediated knock in by 1.7- to 3.5-fold in human iPSCs and by 1.4- to 6-fold in HEK293T cells.[45,47,49]

(3) Cell-cycle-dependent regulation of the activity of programmable nucleases.[49–51] Analogously, HDR-mediated knock-in can be improved by selectively expressing programmable nucleases exactly in the S/G2 phases. Chin and colleagues demonstrated that fusing Geminin residues (aa 1–110) to the C-terminus of Cas9 allows for the cell-cycle-dependent regulation of Cas9 expression, resulting in a 1.9-fold increase in HDR-mediated genome insertion efficiency.[50] Doudna and colleagues observed high efficiency of HDR-mediated knock-in using timed delivery of Cas9-guide RNA ribonucleoprotein complexes across various cell lines and human primary cells.[49]

(4) Modification of donor template DNA.[26,52,53] The lengths of exogenous DNA and homology arms are crucial determinants for HDR-mediated genome insertion. Generally, increasing the lengths of homology arms within a certain range (∼50 bp to >1 kb) can increase HDR efficiency.[26,54,55] However, longer homology arms (>4 kb totally between both arms) do not improve, but rather decrease the frequency of HDR.[56,57] Both circular and linearized donor templates (either dsDNA or ssDNA) can be utilized for HDR-mediated genome insertion (Figure 1A).[58–60] Compared with dsDNA, ssDNA provides higher specificity due to their lower random integration rate and reduced blunt or concatemeric insertion. Chemical modifications of donor templates

Table 1. Comparison of technologies of targeted genome insertion mentioned in this review

Types of knock-in technologies	Genome insertion component	Homology arms	Capability of knock-in	Cell cycle dependence	DSB generation	Genotoxicity	Undesired outcome	Efficiency in mammalian cells
DSB repair-dependent technology								
HRR-mediated knock-in	programmable nuclease (ZFN, TALEN or CRISPR-Cas), donor template (dsDNA or ssDNA)	50–1,000 bp (even longer)	1–10 kb	limited to S/G2 phase	yes	DSB-associated genotoxicity	modest indels and off-target editing[a]	high efficiency
SSA-mediated knock-in	Programmable nuclease (CRISPR-Cas), linearized donor template (dsDNA)	100–600 bp	~2 kb	limited to S/G2 phase	yes[b]	DSB-associated cellular toxicity	relatively low indel and low off-target editing	modest efficiency
NHEJ-mediated knock-in	programmable nuclease (ZFN, TALEN or CRISPR-Cas), linearized donor template (dsDNA)	None	>1 kb	No	Yes	DSB-associated genotoxicity	high indels, inversion of donor DNA, duplication and off-target editing	high efficiency
MMEJ-mediated knock-in	programmable nuclease (ZFN, TALEN or CRISPR-Cas), linearized donor template (dsDNA)	5–40 bp	≤5 kb	G1/early S phase	yes	DSB-associated genotoxicity	high indels and low off-target editing	modest efficiency
Integrase coupled with programmable nuclease								
Integrase	integrase, cargo DNA	none (*attP* X *attB* recombination)	wide range (1 to >100 kb)	no	no	N.A.	off-target edits due to other pseudolanding pads	low efficiency
PASTE	nCas9-RT-BxbI, attachment site-containing gRNA (atgRNA), cargo DNA	none (*attP* X *attB* recombination)	wide range (≤36 kb)	no	no	minimal genotoxicity	combined effect of PE and integrase	low efficiency
Prime editing and paired prime editing								
Prime editing	Cas9 nickase (nCas9)-RT fusion protein, pegRNA	none	~50 bp	no	no	minimal genotoxicity	low indels and off-target editing	modest efficiency
Paired prime editing	nCas9-RT fusion protein, pegRNA pairs	none	~1 kb	no	no	minimal genotoxicity	low indels and off-target editing	modest efficiency
TJ-PE	nCas9-RT fusion protein, template-jumping gRNA (TJ-gRNA)	none	~800 bp	no	no	minimal genotoxicity	low indels and off-target editing	modest efficiency
Transposon and CAST								
Transposon	transposase, cargo DNA	none	wide range (1 to >100 kb)	no	no	N.A.	semi-random transposition	low efficiency
Type I-F CAST	cascade-TniQ, TnsA, TnsB, TnsC, crRNA, cargo DNA	none	wide range (1 to >100 kb)	no	no	N.A.	inversion of donor DNA and low off-target editing	extremely low efficiency
Type V-K CAST	Cas12K, TnsB, TnsC, TniQ, S15, sgRNA, cargo DNA	none	wide range (1 to >100 kb)	no	no	non-specific DNA TnsC filamentation-associated genotoxicity	co-integrate products and high off-target rate	not tested

N.A., not available.
[a]Frequency of off target largely depends on the sgRNA sequences.
[b]Both nCas9 and dCas9 are also used for SSA-mediated knock-ins, which do not generate DSBs.

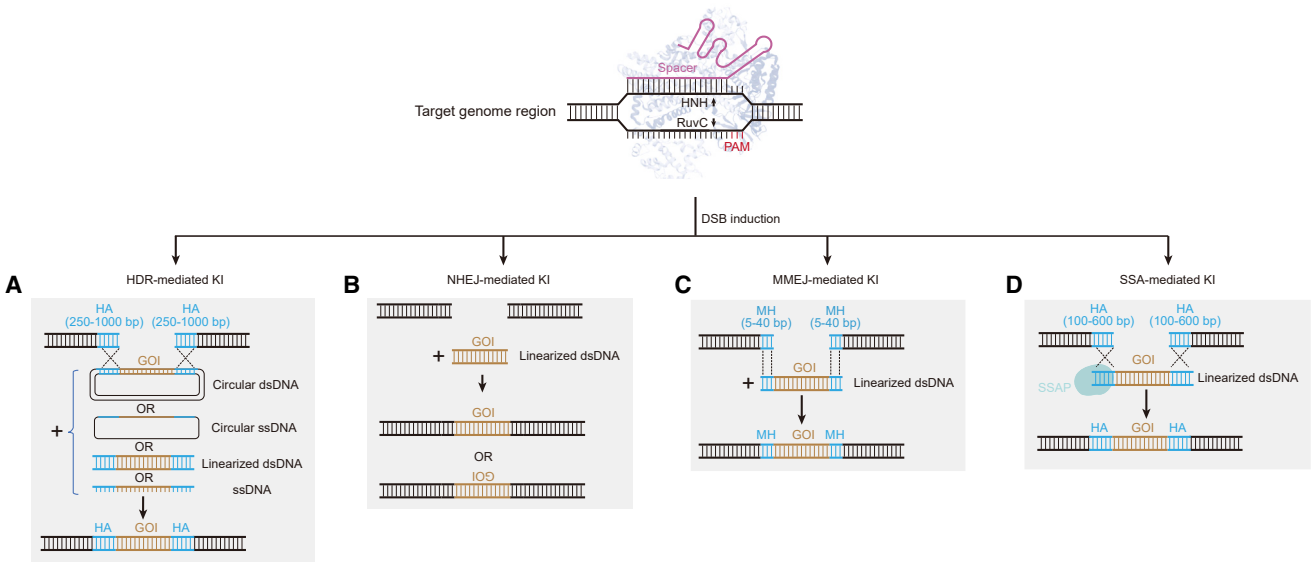

Figure 1. DSBs repair-mediated knock-in strategies

(A) HDR-mediated knock-in is based on homologous recombination (HR) between the long homology arms present in the target genome and donor vector (circular dsDNA, linearized dsDNA or ssDNA). Dashed lines refer to the HR event. (B) NHEJ-mediated knock-in relies on end-joining, either blunt end or sticky end, between donor DNA and target genome around DSBs. (C) MMEJ-mediated knock-in starts requires short microhomology, which starts resection of the microhomology and is followed by annealing of the sequences. (D) SSA-mediated knock-in leverages phage-encoded SSAPs to preform precise recombination which works in a sequence-independent manner. dsDNA, double-stranded DNA; GOI, gene of interest; HA, homology arms; MH, microhomology; ssDNA, single-stranded DNA.

(dsDNA and ssDNA) have also proved effective in enhancing the efficiency and specificity of HDR.[52,53] This is probably due to the improved stability of donor templates and reduced occurrence of blunt or concatemeric insertion into DSBs through the NHEJ repair pathway. Chemical conjugation of programmable nucleases and donor templates can increase local concentrations of DNA templates near target sites, thus increasing knock-in efficiency.[61,62] Marson and colleagues added truncated Cas9 target sequences to the end of the HDR template, which interact with Cas9 ribonucleoproteins to shuttle the template to the nucleus.[63] On average, this design increases the HDR efficiency by approximately 2- to 4-fold.[63] They further developed a hybrid ssDNA HDR templates using a long ssDNA with short regions of dsDNA containing CTS sites on each end, which boosts knock-in efficiency of around 2- to 3-fold relative to dsDNA CTSs.[64] Liang and coworkers developed Long dsDNA with 3′-Overhangs mediated CRISPR Knock-in, which utilizes a donor with hybrid 3′-overhang dsDNA structure for efficient and accurate knock-in of large cargo DNA.[65] To overcome inefficient delivery of cargo DNA into cells, several studies have demonstrated that retrons coupled with CRISPR-Cas9 facilitate precise knock-in via HDR by fusing guide RNA (gRNA) to the 3′ end of retron ncRNA, which can be partially reverse transcribed by retron into a multicopy ssDNA.[66]

NHEJ-mediated knock-in

Since NHEJ is the dominant repair pathway in mammals, NHEJ-mediated knock-in exhibited higher efficiency than that of HDR-mediated insertion (Figure 1B).[67–70] Yang and colleagues devised a method named obligate ligation-gated recombination (ObLiGaRe), which leverages on the NHEJ repair pathway for foreign DNA insertion.[67] ObLiGaRe utilized ZFN and TALEN to generate compatible DSB sticky ends in the target genome and cargo DNA, facilitating the insertion of cargo DNA into the DSB through annealing and ligation of complementary overhangs.[67] Our group further optimized this method by using electroporation to deliver Cas12a/CRISPR RNA (crRNA)/donor dsDNA into human primary cells, thus enabling highly efficient and accurate genome insertion.[71] Belmonte and colleagues reported a CRISPR-Cas9-based knock-in strategy known as homology-independent targeted integration (HITI).[69] HITI utilizes CRISPR-Cas to generate blunt ends in both target and foreign DNA, which are then repaired through NHEJ. Successful applications of HITI have been demonstrated both in *in vitro* and *in vivo*, with the capability of inserting up to 4.6 kb foreign DNA.[69,72,73]

Overall, NHEJ-based knock-in is feasible in both dividing and nondividing cells without significant limitations on donor DNA size. However, NHEJ-mediated knock-in has drawbacks such as frequent indels at both the 5′ and 3′ junctions due to error-prone NHEJ process and potential inverted orientation of inserted foreign DNA.[69,72] These limitations greatly restrict the applications of NHEJ-based knock-in for precise gene editing.

MMEJ-mediated knock-in

MMEJ operates during G1/early S phase, and up to 58% of Cas9-induced DSBs are repaired through the MMEJ pathway.[8] Suzuki and

colleagues devised a method called Precise Integration into Target Chromosome (PITCh), which exploits the MMEJ repair pathway for targeted genome insertion (Figure 1C).[74] PITCH has been extensively applied for site-specific foreign DNA knock-in in human primary cells, zebrafish, and mouse.[52,75,76] Later, a robust knock-in strategy named primed micro-homologues-assisted integration was devised to enhance targeted insertion of donor DNA by using reverse-transcribed single-stranded micro-homologues.[77] This approach achieves knock-in efficiency of up to ~85% in various cell types.[77] However, similar to NHEJ-mediated knock-in techniques, MMEJ-mediated knock-in may harbor random indels at the DSB junctions. Recently, Yang and colleagues devised a homology-mediated end joining (HMEJ)-based strategy that combines CRISPR-Cas9-mediated cleavage of both donor template containing gRNA target sites and approximately 800 bp of homology arms for precise integration.[78] In comparison with MMEJ, HMEJ utilizes longer and potentially more stable homology arms, thereby facilitating efficient integration of foreign DNA. HMEJ-based strategy exhibits higher knock-in efficiency than HR-, NHEJ-, and MMEJ-based strategies in specific cell types and animal embryos.[78]

Single-strand annealing-mediated knock-in

Phage-encoded single-stranded DNA-annealing proteins (SSAPs), such as lambda Bet or its homologue RecT, are enzymes that facilitate precise recombination in prokaryotes.[79,80] Cong and colleagues developed a system called RecT Editor via Designer-Cas9-Initiated Targeting (REDIT) by combining the phage SSAP RecT with a programmable Cas9, which demonstrated five times higher efficiency than HDR-mediated knock-in when using different donor DNAs of kilobase scale (Figure 1D).[81] The enhanced genome insertion efficiency can be attributed to two factors: (1) the REDIT system utilizes both SSAP RecT and the endogenous HDR repair pathway for genome insertion, and (2) the strong affinity of SSAPs for dsDNA or ssDNA enables frequent attachment of donor DNA to the targeted genome when SSAPs are recruited through RNA-guided Cas9. To minimize random indels following DNA cleavage, this group further upgraded the REDIT system by fusing SSAP with catalytically inactive dCas9.[82]

Delivery approaches of donor template DNA

Delivery of donor template in both dsDNA and ssDNA format into cells can be achieved using chemical and physical methods, but these approaches may result in high toxicity, particularly in primary cells.[83,84] Viral vectors, including recombinant adeno-associated virus (rAAV), lentivirus and adenovirus, serve as alternative vehicles for delivering donor templates both *in vitro* and *in vivo* settings.[85–87] Highly efficient knock-in of donor DNA has been reported using rAAV-assisted delivery (e.g., up to 85% knock-in efficiency in human natural killer cells),[86] which is attributed to high stability and availability of donor templates in the nucleus. One limitation of rAAV-based delivery is its relatively low capacity of donor DNA template (<4.7 kb). Complementary to rAAV, lentivirus can carry donor templates up to 8 kb and is capable of efficiently transducing various cell types. Qi and coworkers developed CRISPR for long-fragment integration via pseudovirus, which utilized integrase-deficient lentivirus to insert donor templates flanked by homology arms at specific genomic sites in human primary cells.[85] Adenovirus has also been reported as a delivery vehicle for cargo DNA. Kohn and colleagues successfully used adenovirus 5/35 serotype to deliver the homologous repair donor template encoding HBB to HSPCs, achieving HDR efficiency of up to 5%.[87]

DSB repair-mediated genome insertion has emerged as one of the primary knock-in approaches due to its high efficiency and ease of use, demonstrating remarkable success in preclinical and clinical research. However, targeted genome insertion technologies that rely on DSB repair pathways inevitably lead to indel errors around the DSB junctions, and the efficiencies of foreign DNA knock-in vary greatly depending on cell types and phases of cell cycles.

Integrase coupled with programmable nuclease

Site-specific recombinases (SSRs) have been recognized as valuable tools for genome engineering, as they can mediate excision, integration, inversion and translocation of DNA in living cells.[88] SSRs encompass tyrosine recombinases and serine recombinases based on the nucleophilic amino acid residue.[89] Among these SSRs, integrases, particularly serine integrases, have been extensively used in genome engineering (extensively reviewed elsewhere).[88–91]

Serine integrases catalyze the integration of foreign DNA into bacterial genomes through recombination between *attP* (phage-specific attachment site of approximately 50 bp) and *attB* (bacteria-specific attachment site of approximately 40 bp).[90] Integration can also occur at pseudo *att* sites that share sequence similarity with wild type *att* sites. Successful integration of large foreign DNA (>100 kb) using serine integrase has been achieved in various cell types and animals, despite variable efficiency among different genomic loci.[92–95] Moreover, when multiple pseudo *att* sites exist in the target genome, targeted insertion of foreign DNA at a desired pseudo site becomes challenging.[96] To overcome these limitations, several research groups have utilized direct evolution and structure-guided reprogramming techniques to design recombinase variants with improved integration efficiency and specificity.[97–99] Alternatively, fusion of catalytic domains from recombinases with DNA binding domains from programmable nucleases has proven effective in enhancing both targeting specificity and integration efficiency.[100–102]

Pre-installation of an *att* site into the target genomic site (known as a "landing pad") offers an alternative strategy to facilitate integrase-mediated foreign DNA knock-in. Initially, HDR-mediated knock-in approaches were used to pre-install a landing pad at desired genomic loci.[103,104] Recently, Gootenberg and colleagues developed a novel system, called Programmable Addition via Site-specific Targeting Elements (PASTE), which utilizes a CRISPR-Cas9 nickase fused with both a reverse transcriptase and a serine integrase for targeted integration of desired payloads (Figure 2).[12] Specifically, the PASTE system involves inserting landing sites (~46 bp) through prime editing (as discussed below), followed by recognition of these landing sites and integration of foreign DNA through Cas9-directed integrase.

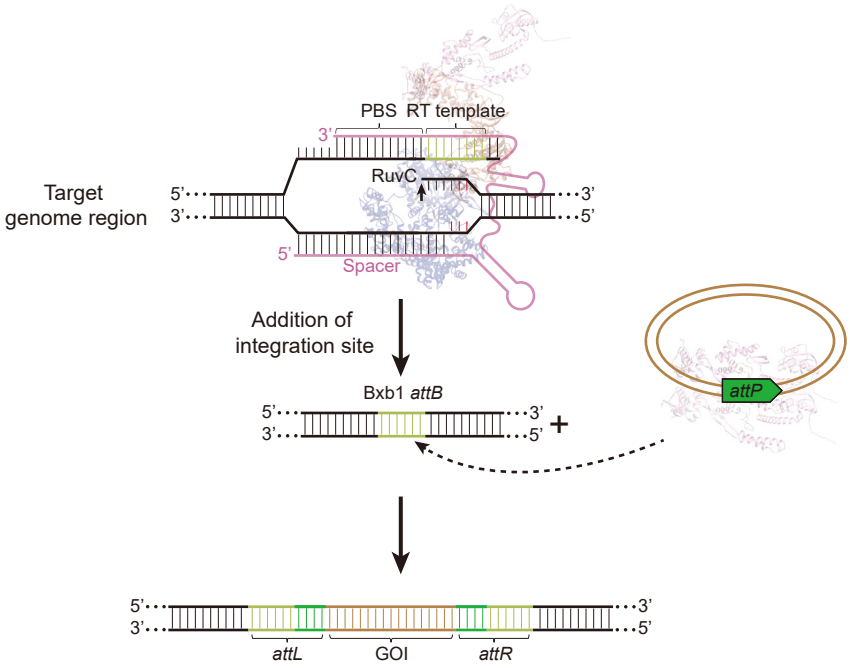

Figure 2. Schematic overview of knock-in using PASTE

The PASTE system involves insertion of landing sites via strategies resembling prime editing or paired prime editing, followed by landing site recognition and integration of external DNA by integrase.

The PASTE system demonstrated cargo DNA integration efficiency of up to 50% in HEK293FT cells, while in K562 cells and human primary T cells, the integration efficiency was only ∼15% and 5%, respectively.[12] A similar strategy was proposed by Liu and coworkers, which utilized twinPE to install the landing pads instead.[10] Additionally, Gao and colleagues introduced the Prime editing-mediated Recombination Of Opportune Targets system, which combines prime editor with plant-optimized recombinases for site-specific integration of DNA fragments in rice.[105]

Collectively, integrases along with derived tools further expand the capabilities of site-specific knock-in by enabling large-size multiplexed gene insertion without relying on DNA repair pathways. Notably, both SSRs and PASTE systems (as well as the CAST systems mentioned below) are not seamless, but leaves genomic scars from split landing pads.

Prime editing and paired prime editing

Recently, Liu and colleagues introduced prime editing, a technique that combines a programmable nickase fused to a reverse transcriptase with an extended prime editing gRNA (pegRNA) to target specific sites and facilitate desired genome edits (Figure 3A).[9] Prime editing enables substitutions, small indels within the living cell genomes, without generating DSB. The mechanism, applications, and limitations of prime editing have been extensively reviewed elsewhere.[106,107] It is worth noting that prime editing can only achieve small foreign DNA knock-ins up to approximately 50 bp in length.[9]

To unlock the potential to program genome insertion of large external DNA using prime editing, several prime editing approaches using two pegRNAs have been reported, including twinPE and genome editing by RTTs partially aligned to each other but non-homologous to target sequences within duo pegRNA (Figure 3B).[10,11] These approaches employ pairs of pegRNA in a PAM-in orientation to template 3′ flaps on opposite strands, where pegRNA pairs can target sites either in close proximity or at greater distances from each other. The 3′ flaps do not need to be fully complementary to each other, but only require 20 nt of overlap on their 3′ ends, which enable efficient knock-in of DNA sequences up to ∼150 bp.[10,11] Inspired by retrotransposon mechanism, Xue and colleagues developed template-jumping prime editing (TJ-PE) for large DNA fragment insertion using a single TJ-pegRNA (Figure 3C).[108] TJ-pegRNA harbors the insertion sequence as well as two primer binding sites (PBSs), with each PBS matching a nicking sgRNA site on opposite strands, which can template synthesis of both strands of the inserted sequences. TJ-PE exhibited site-specific knock-in of up to ∼800 bp foreign DNA both *in vitro* and *in vivo*.[108]

Overall, prime editing and paired prime editing hold great potential for targeted and scarless genome insertion. However, the maximum length of inserted foreign DNA is ∼50 bp for prime editing and ∼800 bp for paired prime editing or TJ-PE, thereby restricting their applications in biological research and translational research. Further exploration of prime editing derived tools, such as combining prime editing with other knock-in techniques, is warranted to enhance the efficiency of genome insertion of even longer donor DNA.

Transposable elements and CAST

Transposable elements

Transposable elements (TEs) are DNA sequences capable of changing their positions within a genome. TEs can be divided into two major classes based on their transposition mechanisms: retrotransposons that mobilize through a copy-and-paste mechanism and DNA transposons that mobilize via a cut-and-paste or peel-and-paste mechanism.[109] The principles underlying the functionality of retrotransposons have been adopted by prime editing, as discussed above. In this section, we focus on DNA transposons.

The fundamental components of a DNA transposon consist of a left end (LE) sequence, a right end (RE) sequence, and a central DNA fragment encoding a transposase. During transposition, the transposase initially binds to the LE and RE to form a complex, which is then excised

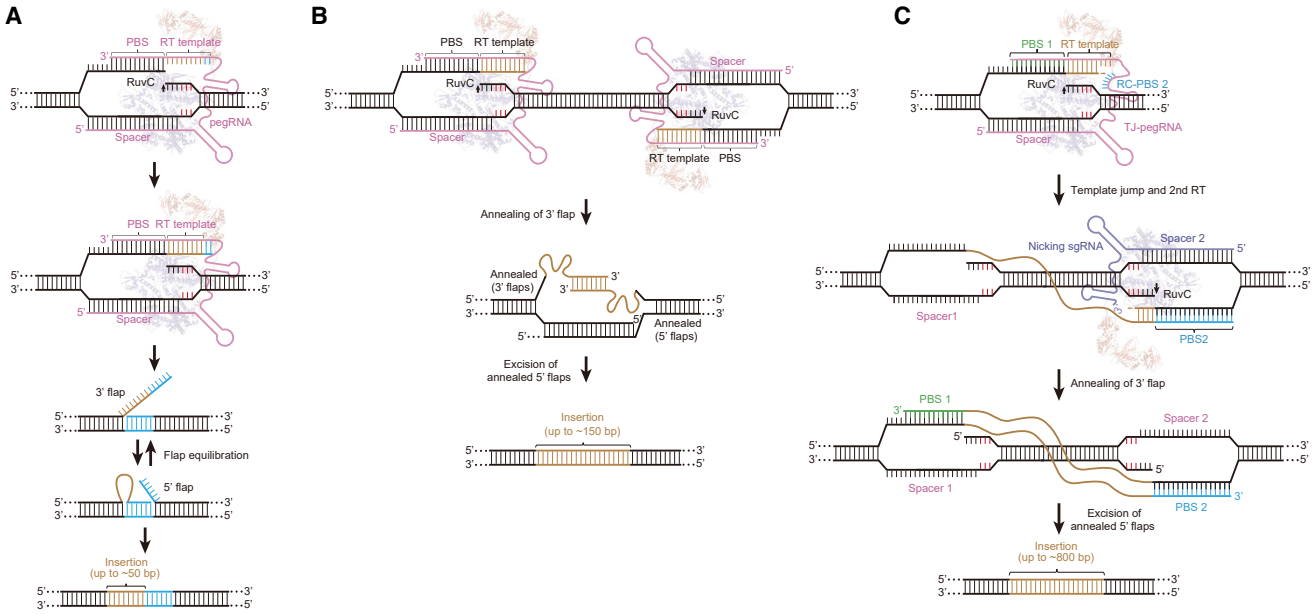

Figure 3. Schematic overview of prime editing and paired prime editing

(A) Overview of prime editing. A prime editing complex consists of a prime editor (an RNA-guided DNA nicking domain, such as Cas9 nickase, fused to and reverse transcriptase domain) and a pegRNA. The prime editing complex binds the target DNA and nicks the non-target strand, and the resulting 3′ end hybridizes to the PBS sequence, then initiates reverse transcription of new DNA containing the desired insertion fragment (up to ∼50 bp) using the template of the pegRNA. Equilibration between the edited 3′ flap and the unedited 5′ flap, excision of the unedited 5′ flap, and DNA repair results in stably edited DNA. (B) Overview of paired prime editing. Prime editing with paired pegRNAs that template two 3′ DNA flaps containing the edit. Annealing of the fully or partially complementary flaps, excision of the original genomic duplex, and DNA repair mediates large DNA fragment insertion (∼1 kb). (C) Schematic of TJ-PE. In contrast with pegRNA designed for prime editing, the TJ-pegRNA consists of two PBSs, which initiates two rounds of reverse transcription sequentially thus enabling insertion of long external DNA (up to ∼800 bp). RT template, reverse transcription template.

from the donor DNA and inserted into a new location by the transposase. To date, numerous types of DNA transposons have been identified and further engineered for genome insertion.[110–112] Despite their ability to carry large cargo DNA (up to ∼100 kb) and achieve high integration efficiency, transposases cannot mediate site-specific integration, but instead do so randomly. Tremendous efforts have been made to fuse transposases, such as *Sleeping Beauty* and *PiggyBac*, with active or catalytically inactive programmable nucleases or their DNA binding domains, to facilitate targeted genome integration that would otherwise be challenging for transposases.[113–116] These fusion systems have demonstrated efficient programmable insertion of foreign DNA in various cell types and animal models.

CAST

Historically, transposons, such as Tn7 and non-Tn7-like transposons, have also co-opted nuclease-deficient type I or type V CRISPR-Cas systems to catalyze RNA-guided integration of mobile genetic elements into target sites in the genome.[117] Tn7-like transposons possess characteristic left- and right-end sequences along with five Tns genes (*TnsA–E*) that collectively encode a heteromeric transposase. TnsA and TnsB are transposases that excise the TE via coordinated DSB; TnsB also catalyzes DNA integration; TnsD and TnsE constitute mutually exclusive targeting factors that recognize specific DNA insertion sites; and TnsC is an ATPase that communicates between TnsA/TnsB and TnsD or TnsE.[13,14]

Subtype I-F CAST

Recently, Sternberg and co-workers reported the type IFCRISPR effector-based Tn7-like transposon system (*V. cholerae* Tn*6677*) (Figure 4A), showing the ability to INsert Transposable Elements by Guide RNA-Assisted TargEting (INTEGRATE).[13] The structures of type I-F CAST and mechanisms underlying target DNA recognition and cargo transposition have been extensively investigated elsewhere.[13,118–120] To utilize *V. cholerae* Tn*6677* for programmable genome insertion, plasmids encoding components of the *V. cholerae* Tn*6677* were designed and delivered into *E. coli*, including a mini-transposon donor, the tnsA-tnsB-tnsC operon, and the tniQ-cas8-cas7-cas6 operon alongside a synthetic crRNA targeting a specific genomic site. This three-plasmid expression system allows site-specific integration of foreign DNA (up to ∼10 kb) into the bacteria genome, albeit integration efficiency decaying dramatically with longer cargos (>1 kb).[13] The INTEGRATE system was further streamlined by assembling the components of the transposon-associated and CRISPR-Cas systems into one single expression vector called pSPIN, achieving highly efficient and accurate knock-in of foreign DNA up to 10 kb in the bacteria genome while enabling multiplexed knock-in at different genomic loci.[121] In addition to *V. cholerae* Tn*6677*, other CRISPR effector-based Tn-7-like transposons belonging to type I-B, type I-C, type I-D, type I-E, and type I-F have also been reported.[13,14,117,122–124]

Programmable knock-in of large DNA sequences in mammalian cells using the type I-F CAST has also been explored.[125] Sternberg

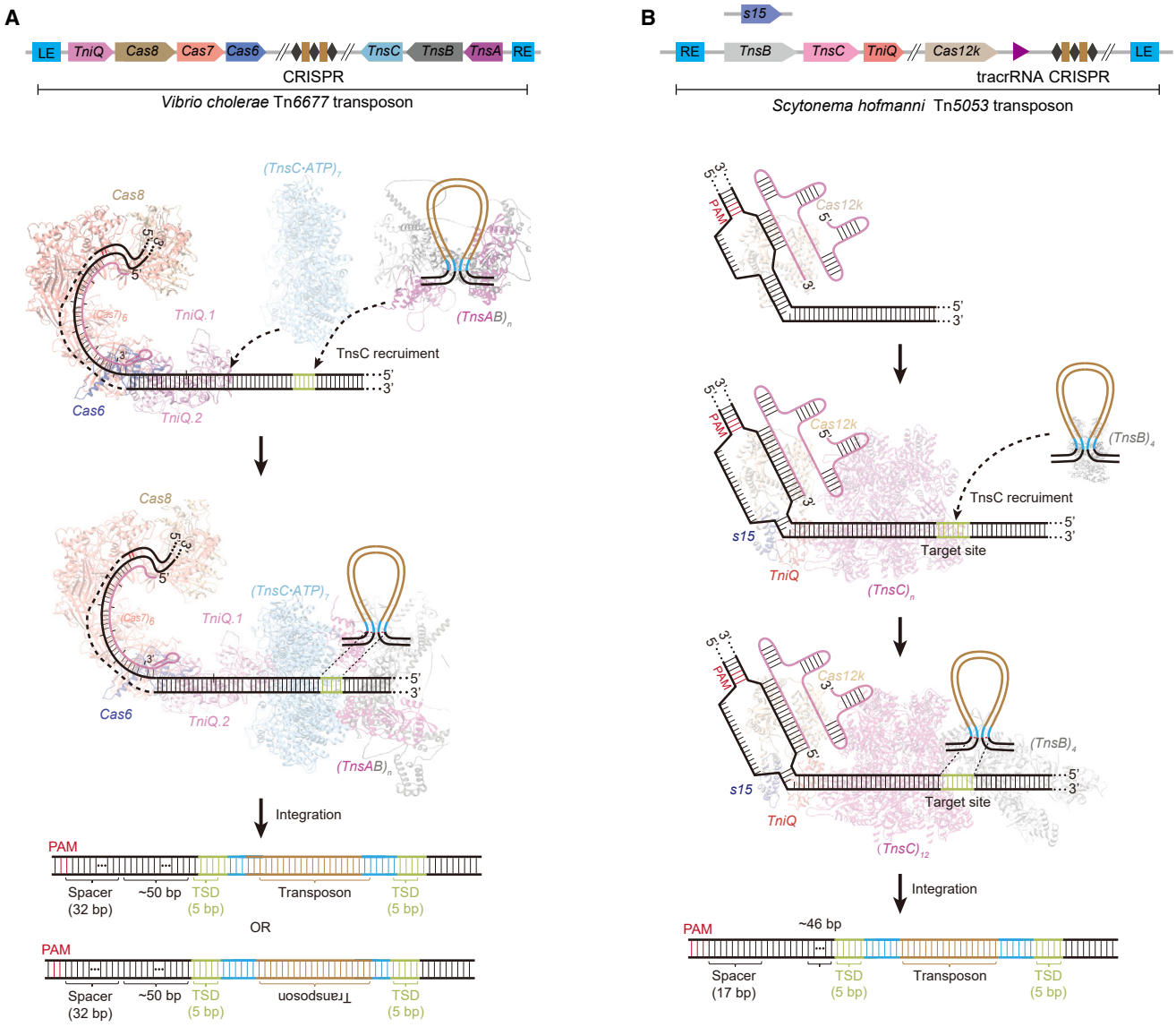

Figure 4. Schematic overview of CASTs

(A) Example of the subtype I-F CASTs. The *V. cholerae* Tn*6677* transposon encodes a TniQ-Cascade co-complex that uses crRNA to target specific genomic sites, while TniQ can further recruit the non-sequence-specific DNA-binding protein TnsC. The transposon itself is bound and cleaved on the 5′ and 3′ end of the transposon by TnsA and TnsB, respectively. The n-transposon DNA complex (TnsAB) is recruited to the target site by TnsC, and finally the transposon is transposed into the genome at a fixed distance downstream of crRNA targeting site. (B) Example of the subtype V-K CAST. The *Scytonema hofmanni* Tn*5053* transposon encodes a catalytically inactive Cas12k protein, which in association with a crRNA-tracrRNA dual gRNA binds target DNA to form a partial R-loop structure. Full R-loop formation occurs upon recruitment of S15, TniQ, and TnsC. The resulting TnsC filament provides a recruitment platform for TnsB, which catalyzes transposon DNA insertion.

and co-workers screened 16 type I-F CAST systems and characterized two diverse systems from *V. cholerae* and *Pseudoalteromonas* that demonstrated genome insertion efficiency of up to ~2% across tested genomic sites in mammalian cells.[125] This work provides a promising starting point for eukaryotic genome engineering using CAST, offering an opportunity for site-specific DNA integration without the need for DSBs in the target DNA, homology arms in the donor DNA, and host DNA repair factors.

Subtype V-K CAST

Another subtype of CAST from cyanobacteria *Scytonema hofmanni* (ShCAST) and *Anabaena cylindrica* has been recently described by Zhang and coworkers, which consists of Tn7-like transposase and the type V-K CRISPR effector Cas12k (Figure 4B).[14] The Cas12k effector possesses a naturally inactivated RuvC-like nuclease domain and can specifically target genomic sites of 24 bp length immediately after a 5′ *NGTN* PAM. The structures of type V-K CAST and mechanisms underlying target DNA recognition and cargo transposition

have been summarized elsewhere.[126–130] ShCAST catalyzes RNA-guided DNA transposition by unidirectionally inserting DNA segments 60–66 bp downstream of the protospacer, achieving an efficiency as high as 80% for foreign DNA insertion into the *E. coli* genome.[14] In contrast with type I-F CAST, type V-K CAST only encodes TnsB transposase that nicks at the 3′ end. As a result, the type V-K CAST is prone to generating a mixture of simple insertion and cointegrate insertions through replicative transposition.[131] To improve integration product purity mediated by type V-K CAST, Kleinstiver and colleagues fused a nicking homing endonuclease to TnsB (named HELIX) to restore its 5′ nicking capability observed in type I-F CAST system by TnsA.[132] HELIX enables cut-and-paste DNA insertion with up to 99.4% simple insertion product purity while maintaining robust integration efficiencies at genomic targets in *E. coli*.[132]

Overall, the CAST system serves as an ideal technology for efficient single-step genome insertion of large DNA cargos with high specificity and programmability, without relying on DSBs or HR. Although applications of the CAST system in eukaryotes are still in their infancy, they present new opportunities for circumventing the challenges faced by conventional gene knock-in methods.

Applications of genome insertion technologies in translational medicine

Recent advancements in programmable insertion technologies have enabled highly efficient and precise site-specific knock-in of genetic materials, leading to successful applications in cell models, organoids and animal models.[133,134] These models play a crucial role in facilitating preclinical safety and efficacy evaluation of cellular and gene therapy products. The OMIM database has curated ∼4,000 genetic diseases caused by recessive loss-of-function mutations and ∼250 genetic diseases caused by dominant-negative mutations. Targeted knock-in techniques can introduce normal genes or DNA fragments into mutant organisms to restore the normal function of mutant genes, making them particularly relevant for gene therapy approaches of these genetic diseases (Table S2). For instance, hemophilia is an inherited X-linked monogenic bleeding disorder resulting from loss-of-function mutations in the FVIII or FIX gene.[135] Targeted insertion of FIX cDNA exons 2–8 into endogenous intron 1 of the FIX gene locus can efficiently decrease the bleeding time.[136] Similarly, knock-in technology has been used to correct cystic fibrosis transmembrane conductor receptor (CFTR) mutations that cause cystic fibrosis. Supplementation of wild-type *CFTR* genes restored standard swelling rates and regained sensitivity to channel inhibitors.[137] Other examples include sickle cell disease and familial hypercholesterolemia,[1,2] where targeted knock-in of normal genes demonstrated satisfactory preclinical and clinical outcomes.

Current manufacturing protocols for CAR-T cell and other tumor immune cells involve the use of lentiviral transduction to introduce chimeric receptor sequences. However, lentiviral transduction may raise the possibility of malignant transformation of engineered CAR-T cells due to insertional disruption of tumor suppressor genes. In addition, expression levels of CAR transgenes may vary greatly by integration into different genomic loci, thus leading to sub-optimal therapeutic effects.[138] To address these concerns related to lentiviral transduction, programmable genome insertion techniques can be used to place the CAR transgene under the control of a predetermined endogenous promoter (Table S2).[3,139] These newly designed CAR-T cells exhibited uniform expression of CAR transgenes, decreased exhaustion, and increased antitumor efficiency. In summary, knock-in technologies have made significant advancements in preclinical and clinical cellular and gene therapy. Further efforts for thorough safety evaluation would greatly facilitate the utilization of knock-in technologies in future translational medicine.

CONCLUSIONS AND PERSPECTIVES

Over the past decade, genome editing technologies have made exponential advancements with unprecedented success in fundamental biological studies and therapeutic applications. Programmable genome insertion has been widely utilized for tagging endogenous genes, developing transgenic animal models and overexpressing therapeutic gene products. The ability to insert full exons or entire genes enables therapeutic correction of genetic diseases through the insertion of full-length functional genes at native loci, a feasible strategy for treating patients caused by both recessive loss of function mutations and dominant negative mutations.[140–142] Beyond direct correction of hereditary diseases, gene insertion provides a promising avenue for cell therapies. Efficient integration of engineered transgenes such as CARs at specific loci can produce improved therapeutic products compared with random integration.[3,139,143] Despite holding great promise, targeted insertion of large DNA fragment (>1 kb) remains challenging.

The number of strategies developed for targeted genome insertion is ever-expanding. Since these programmable knock-in technologies come with their own capabilities and limitations, a choice between diverse methods has to be made when attempting site-specific genome insertion. The ideal programmable genome insertion techniques should be (1) compact in size allowing easy delivery into cells; (2) efficient in knocking-in large cargo DNA across diverse cell types; (3) accurate with minimal undesired editing outcomes; and (4) tunable to user-defined parameters for various applications.

Efforts are still required to further enhance the efficacy and safety of current knock-in systems, especially in mammalian cells. Addressing these challenges will necessitate a focus on several key areas (1) elucidating the mechanisms underlying both on-target insertion and off-target effects and (2) engineering existing knock-in techniques and developing novel DNA genome insertion techniques. Through continuous efforts in the development and optimization of genome insertion technologies, we anticipate significant breakthroughs could be achieved in the coming decade.

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

None.

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