



CRISPR-Cas gene perturbation and editing in human Pluripotent Stem Cells

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3 **TITLE: CRISPR-Cas gene perturbation and editing in human Pluripotent Stem Cells**

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Abstract

Directing the fates of human Pluripotent Stem Cells (hPSC) to generate a multitude of differentiated cell types allows the study of the genetic regulation of human development and disease. The translational potential of hPSC is maximised by exploiting clustered regularly interspersed short palindromic repeats (CRISPR) to permanently or reversibly silence or activate genes with spatial and temporal precision. Here, we summarise the increasingly refined and diverse CRISPR toolkit for the latter forms of gene perturbation in hPSC, and their downstream applications. We discuss newer methods to efficiently install edits with single nucleotide resolution and describe pooled CRISPR screens as a powerful means of unbiased discovery of genes associated with a phenotype of interest. Last, we discuss the potential of these combined technologies in the treatment of hitherto intractable human diseases and the challenges to their implementation in the clinic.

Introduction

Progress in understanding human development and disease has largely depended on the successful translation of discoveries from lower mammalian models. While this approach has undoubtedly benefited human health, the workings of human-specific gene regulatory networks that encode the numerous distinct human cellular lineages, and determine their physiology remain poorly understood. These species differences also compromise accurate modelling of human diseases in laboratory animals, commonly the mouse, which hampers the development of new treatments.¹ Furthermore, the mechanisms that confer pluripotency and allow differentiated cells to acquire cell states corresponding to the inception of development, are relevant to the pathogenesis of cancer. To address these issues, two important and inter-related goals need to be met, on which hinge improved disease modelling and therapeutic advances.

First, human cellular models need to be closely aligned to their *in vivo* counterparts, but genetic and phenotypic divergence of cultured cells limits their usefulness.^{2, 3} This gap cannot be filled easily by using primary cells from humans because of their limited capacity for expansion, and the difficulty of obtaining certain disease relevant cell types, such as neurons. Human pluripotent stem cell lines namely Embryonic Stem Cells (ESC) and induced Pluripotent Stem Cells (iPSC) are self-renewing and can overcome some of the major obstacles in probing human development and disease. *In vivo*, pluripotency is a transient cellular state during early embryogenesis instructed by a core set of transcription factors that are also capable of inducing pluripotency in differentiated cells, commonly fibroblasts.⁴ By applying differentiation protocols pluripotent cells can be converted into a variety of cell lineages, tissues and even organ-like structures with 3D architectures.⁵ These cells and their differentiated progeny are undergoing clinical trials aiming to regenerate or replace tissues and cell types.^{6, 7} The use of iPSC enables the generation of autologous cell types, overcoming immunological rejection which is the main obstacle in current cell and tissue replacement therapies. Coupled with precise and scalable gene editing to maximise their utility, human iPSC (hiPSC)-derived cells and tissues present exciting opportunities to treat genetic diseases such as haemophilia, β -thalassemia, and Duchenne Muscular dystrophy (DMD), to name but a few.⁸

Second, gene perturbation technologies that are functional in biologically relevant cell types are needed to probe genetic contributions to cellular physiology and pathology and opens new avenues for treating currently intractable diseases.^{9, 10} To investigate disease mechanisms, using isogenic hiPSC from a single parental donor with the intention of engineering a diverse set of disease-

causing mutations, would avoid the confounding effects of different genetic backgrounds on the cellular phenotype. The challenge that must first be overcome is to install the desired genetic modification efficiently and accurately in the genome of hPSC.

In hPSC, editing efficiency for precise genome changes is typically low,^{11, 12} which necessitates testing and adapting gene editing technologies so they work optimally in this cell type. Even when transgenes have been successfully integrated, they could become silenced upon differentiation.¹³ hPSC require colony culture conditions, which makes it harder to sort single cells for selection, and subcloning is essential to confirm the presence of the desired edit. Like other cultured cells, hPSC can be predisposed to somatic mutations that confer a selective growth advantage. The use of hPSC is predicated on the presence of a normal karyotype, which is why they are deemed to be more informative for disease modelling than cancer cell lines. However, large chromosomal structural changes often involving partial or complete gains of chromosomes 12, 17, 20 and X are well-recognised on karyotyping hESC and hPSC, as are smaller structural variants, including gains in 20q11.21.¹⁴⁻¹⁶ The potential for oncogenic transformation posed by these mutations is, to a degree, offset by the high sensitivity of edited hPSC to cell cycle arrest or apoptosis, elicited by a DNA damage response to DNA cleavage that requires the tumour suppressor gene, *TP53*.^{17, 18} Even when gene editing in PSC has been successful, determining the function of a gene of interest in a fully differentiated cell type could be hindered by additional roles for the same gene at proximal points in the lineage. This problem cannot be easily circumvented, since attempting to install the desired edit in non-dividing, terminally differentiated cells is often not feasible.

Here, we describe the suite of CRISPR/Cas tools available to perturb the coding fraction of the human genome, in human non-PSC lines, which are invariably cancer cell lines, and concurrently highlight the approaches which have been successful in hPSC. Identifying genome editing approaches that are functional and efficient in hPSC specifically, is important because of the lower efficiency of genome editing, in general, in this cell type compared to human cancer cell lines. We discuss the application of these technologies to hPSC genome engineering in health and disease using illustrative examples, highlight some of the difficulties encountered and options to address these. Older genome editing approaches, in particular meganucleases, targeted zinc finger nucleases (ZFN) and transcription activator-like effector (TALE) proteins have been extensively reviewed elsewhere and are not discussed here.

CRISPR genome editing and its detection

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3 Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) are bacterial DNA
4 sequences that represent a cornerstone of an adaptive immune system in bacteria to target foreign
5 viral DNA.¹⁹ This pathway has been modified and repurposed to enable precise genome editing in
6 mammalian cell lines, including hPSC.
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10 **Overview of CRISPR-Cas9 mechanism and repair of DNA breaks**

11 The Class 2, type-II CRISPR/Cas is most widely used and is based on a CRISPR-associated protein,
12 Cas9 derived from *Streptococcus pyogenes* (SpCas9). Cas9 is directed by a guide RNA (gRNA)
13 incorporating CRISPR targeting (crRNA) and trans-activating RNA (tracrRNA) components, to
14 target a defined, short double stranded DNA sequence.²⁰⁻²² Upon gRNA loading by Cas9, the
15 universal tracrRNA serves as a scaffold that enables activation of the two Cas9 endonuclease
16 domains, RuvC and HNH. The crRNA consists of a constant region (16-22 nucleotides) required for
17 annealing to the 5' end of the tracrRNA and a variable or "spacer" region (20 nucleotides), which
18 determines gene targeting specificity and is homologous to the double-stranded DNA (dsDNA)
19 target.
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23 The genomic determinant of Cas9 targeting specificity is a twenty nucleotide protospacer sequence
24 proximal (5') to a three nucleotide protospacer adjacent motif (PAM) consisting of the sequence
25 NGG (where N is any nucleotide). Upon Cas9 binding, a transient RNA-DNA helix (R-loop) forms
26 with the opposite, target strand, which is followed by a nuclease-induced double strand break (DSB)
27 in the DNA sequence. DSBs are repaired in two major ways: non-homologous end joining (NHEJ)
28 and homology-directed repair (HDR) (Fig. 1). NHEJ repair is mainly active in G1 of the cell cycle,
29 is error prone and leads to small insertions or deletions (indels) that introduce frameshifts, or
30 premature stop codons (Fig. 1A). Nonsense-mediated mRNA decay of truncated or misspliced
31 mRNA products transcribed from the edited locus prevents translation of a functional protein
32 product, which is often the desired outcome. HDR precisely reconstructs the original genomic
33 sequence by using donor templates with long homologous stretches of DNA (Fig. 1A). HDR occurs
34 predominantly in G2/S-phase, which restricts HDR to actively dividing cells. In comparison to
35 NHEJ, DNA repair by HDR is relatively inefficient. However, introducing a DSB at the targeted
36 site greatly improves HDR-mediated gene editing efficiencies.²³⁻²⁵
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54 Homology-mediated end joining (HMEJ) is fundamentally similar to HDR and uses intrinsic
55 regions of DNA sequence homology a few hundred base pairs in length that are revealed after
56 resection to anneal and repair the DNA after a DSB (Fig. 1A).^{26, 27} Considerably shorter regions of
57 homology (2 – 25 nucleotides) can also be used to repair DSBs and this DNA repair mechanism
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related to HMEJ is termed microhomology-mediated end joining (MMEJ) (Fig. 1A).^{26, 28} DNA repair through the HMEJ/MMEJ pathways results in deletion of the genomic sequence between the two microhomology regions and has more predictable outcomes than NHEJ. This predictability has been exploited in order to more efficiently generate loss- or gain-of-function disease models.^{29, 30} The HMEJ/MMEJ pathways can also be exploited for knock-in of DNA sequence by providing a DNA template with short homology arms. Moreover, HMEJ activity extends from M to early S phase, and so encompasses most of the cell cycle,³¹ making this mode of DNA repair an efficient alternative to HDR for precise knock-in. All these DNA repair pathways have been exploited for mutation, correction or knock-in of DNA sequences into the human genome and are discussed further in this review (Fig. 1B and Table 1).

Detection of gene editing

Profiling gene edits, which include nucleotide substitutions, insertions and deletions (indels) and knock-ins is important to verify the occurrence of an editing event, its functional consequences (for example, gene knockout) and for therapeutic applications. With respect to the the latter consideration, the opposite outcome, restoration of gene function is, in some instances, also important. For example, gRNA designs that lead to a single base pair insertion can restore expression of the protein dystrophin that is deficient in DMD through a process of gene ‘reframing’.³²

Genotyping of edits relies on the detection of polymerase chain reaction (PCR)-generated amplicons that can detect DNA alterations with single base resolution. For indels, current detection methods have a sensitivity limit of 0.1%, meaning that a single editing event can be detected in as few as 500 diploid cells (1 in 1000 template chromosomes). As such, several thousand input cells are needed for reliable detection. PCR based methods including the highly sensitive digital droplet PCR can be used that are suitable for the detection of both small indels and substitutions.³³⁻³⁵ Initially, if desired, less sensitive screening approaches such as agarose gel electrophoresis of amplicons, or restriction fragment length polymorphism (RFLP) analysis can be used to detect edits in clonal hPSC cell lines.^{25, 36} Other enzymatic methods include the widely-used enzyme mismatch cleavage assay.³⁷ In order to reveal the nature of the edit, amplicon Sanger sequencing,³⁸ or next generation sequencing (NGS) coupled with bioinformatic analysis is required.³⁹⁻⁴¹

Optimising knock-in success by HDR

In mice, precise transgene integration is routinely achieved through homologous recombination-mediated gene targeting, which is further enhanced by CRISPR.⁴² In contrast to mouse,

homologous recombination in hPSC is very inefficient,⁴³⁻⁴⁵ and was rarely attempted before the advent of nuclease technologies (zinc fingers, TALENS and then CRISPR) to improve precise integration rates. The reasons for this species disparity are likely multiple, and direct comparisons are complicated by the fact that mouse ESC represent a slightly earlier (preimplantation) ‘naïve’ developmental stage than hPSC, which represent a postimplantation, ‘primed’ state.¹² In the following sections we review the factors that influence the success of homology-directed repair. Once a transgene has been successfully knocked-in, it is important to recognise that only one allele might be tagged. The untagged allele could be left with inactivating indels because of dsDNA breaks without recombination, which can confound downstream analysis of cellular phenotypes.

dsDNA templates for precise transgene knock-in by HDR repair

In human embryos, injection of Cas9 mRNA, gRNA and a linearised homologous donor DNA template devoid of vector sequences improved precise knock-in efficiency up to twelve-fold compared to earlier homologous recombination-based methods.⁴² When using dsDNA as the donor template for ‘scarless’ HDR editing in hPSC and human non-PSC, screening for random integration events of the transgene cassette is important.^{46, 47} Furthermore, even if HDR has been successful, the CRISPR nuclease could recut the modified allele to generate indels.⁴⁸ To avoid re-cutting of the newly installed edit, CRISPR blocking silent mutations can be introduced in the spacer or PAM sequence of the donor template,^{48, 49} or truncated guides that block the further activation of Cas9 can be used.^{50, 51} In one example, the workflow combined positive and negative selection steps in two sequential rounds of genome editing to remove cells with unintended indel edits in the targeted gene, or cells with random integrants (Fig. 2A - C).⁴⁶ These enrichment steps were reported to produce 100% correct editing of enriched hPSC clones and is a robust method for the generation of multiple isogenic clones from a single line. Potential difficulties include structural genomic changes, repeated rounds of selection and targeting which are time consuming and labour intensive, and cloning of long (~400 bp) homology arms.⁴⁶ A shorter protocol, which led to a five-fold improvement in HDR efficiency in primary human T-cells involved tethering Cas9 to the repair template, and demonstrated the importance of co-localising all components required for gene knock-in within the nucleus.⁵²

ssDNA templates promote HDR repair

Compared to dsDNA, single-stranded DNA (ssDNA, also termed single-stranded donor oligonucleotides (ssODN)) that is chemically modified to enhance intracellular stability, is less toxic to cultured mammalian cells, avoids unwanted integration at off-target sites, and integrates more efficiently.^{47, 53, 54} ssDNA, rather than dsDNA donors have increasingly been used as the template structure when attempting to integrate heterologous DNA sequences to generate tagged

proteins in hPSC, or for antibiotic selection.^{55, 56} For integration of large reporter cassettes, in human non-PSC, repair by ssDNA requires long homology arms,^{47, 55, 57} and this is also likely to be the case in hPSC, which awaits further study. As with dsDNA, tethering the repair template to the Cas9 protein or to the gRNA⁵⁸⁻⁶³ enhances the efficiency of precise reporter integration in mammalian stem cells including hPSC.⁵⁹ Therefore, for repair templates consisting of either ssDNA or dsDNA⁵² recruitment to the genome editing site appears to be one of the rate-limiting factors for HDR. In contrast to hiPSC, human zygotes can dispense with an ssODN template to correct heterozygous gene mutations, relying instead on the endogenous WT allele for homology-dependent repair.⁶⁴

Enhancing HDR by cell cycle regulation or CRISPR co-selection

Genetic control of cell cycle determinants, engineered regulation of Cas9/gRNA complex activity, or chemical treatment aims to enhance HDR and/or inhibit NHEJ repair in human cell lines including hPSC.⁶⁵⁻⁷² Transient p53 inhibition boosts precision HDR genome editing in hPSC.^{17, 18} Furthermore, hPSC lacking p53 function are capable of self-renewal and can still be differentiated to a variety of specialised cell types, including neuroepithelium and gut epithelium.⁷³ Against this approach must be weighed the greater risk of polyploid and aneuploid cells associated with loss of *TP53*.⁷⁴ To avoid compromising genomic integrity, Cas9 activity can be constrained to the S/G2 phase by tethering the Cas9/gRNA complex to proteins with periodic expression during the cell cycle, or to key HDR regulators.⁷⁵⁻⁷⁷ Small molecules can enhance HDR by enriching cells in the S/G2 phase,⁶⁶ but they could have undesirable pleiotropic effects on the integrity of hPSCs, particularly when they are intended for clinical use.⁷⁸ An alternative approach takes advantage of the inherently different cell cycle kinetics of 'primed' versus 'naïve' hPSC. The former cell state is associated with a longer S/G2 phase that results in higher rates of homology-directed repair of CRISPR-Cas9 induced double-strand breaks.⁷⁹

The enrichment of correctly targeted cells, so-called positive selection makes it far easier to ascertain if the desired genetic edit has been introduced. A classic approach is to knock-out the hypoxanthine phosphoribosyl transferase (*HPRT*) gene, which renders cells harbouring this mutation resistant to an otherwise lethal drug, 6-thioguanine (6-TG).⁸⁰ CRISPR-Cas can be used to induce the *HPRT* mutation and cells surviving in the presence of 6-TG are much more susceptible to additional targeted mutations, a strategy termed co-selection.⁸¹ Along similar lines, recent studies employ co-selection using other universally expressed cell-intrinsic proteins, which, if mutated, render hiPSC and human non-PSC resistant to distinct, normally lethal drugs or toxins.⁸²⁻⁸⁴ Co-selection thus enriches for simultaneous editing at a second desired, independent genomic site and

could supplant antibiotic selection for cell-based therapies, avoiding the introduction of potentially immunogenic bacterial proteins into cells.

Gene knock-in by MMEJ and NHEJ

HDR avoids disrupting gene regulation in the vicinity of the insertion site through precise reconstitution of the genomic sequence flanking the transgene. However, its low efficiency, particularly in hPSC has motivated the investigation of other forms of DNA repair to achieve the same goal. Efficient knock-in of recombinant DNA in human non-PSC, can also be achieved using MMEJ, which in one version, termed Precise Integration into Target Chromosome (PITCh) used MMEJ microhomologies of ~20 bp.⁸⁵ These short sequences which flank the transgenic knock-in cassette are readily added by PCR to a plasmid backbone and avoid the need for cloning long homology arms used in HDR repair. Two gRNAs, one for genomic target recognition and one PITCh gRNA that recognises identical plasmid sequences close to the microhomology sites, facilitate the insertion of the donor sequence by MMEJ (Fig. 3A). The reliable nature of the junctions produced by PITCh was reported to result in more consistent editing outcomes. This is especially advantageous for clinical translation of hPSC technologies, or when it is important that heterologous DNA sequences such as selection markers are eventually removed in a ‘scarless’ manner.⁸⁶ CRISPR technologies have also taken advantage of endogenous microhomologies implicated in human diseases to facilitate improved disease models. These microhomologies flank genomic loci linked to pathogenic copy-number variations and oncogenic chromosomal rearrangements.³⁰ Artificially targeting a small sample of these loci with gRNAs recapitulated human disease mutations in hiPSC. By pairing hiPSC targeted in this way with isogenic controls that have the same genetic background, more realistic disease models can be generated for drug screens and functional studies.

In the right experimental conditions, NHEJ can also generate precise, functional transgene insertions. CRISPaint and the closely related CRISPR-HOT were developed to precisely integrate large reporter tags in endogenous proteins.^{74, 87} In this system, two gRNAs are used; one to direct Cas9 cleavage of genomic DNA at the desired location in a gene of interest, and a second, frame selector gRNA to target a co-transfected plasmid encoding a fluorescent reporter. Different frame selector gRNAs enable cleavage of the reporter-bearing plasmid in all possible reading frames so that the reporter gene is inserted in-frame (Fig. 3B).⁸⁷ When tested in human hepatocyte and intestinal organoids derived from adult stem cells, highly efficient, precise knock-in efficiency averaging 30-40% was reported.⁷⁴. An undesirable consequence of tagging a gene in this way is the

introduction of a strong poly-A signal that can interfere with post-transcriptional regulation by the endogenous 3' untranslated region (3'-UTR). Furthermore, plasmid backbone integration downstream of the gene of interest could induce epigenetic changes that lead to loss of physiological gene expression.⁸⁷ Although the latter outcome can be mitigated by supplying donor DNA as minicircle DNA that is stripped of the plasmid backbone,⁸⁸ CRISPaint and CRISPR-HOT are perhaps better suited for simply marking cells that express a gene of interest. Overall, MMEJ has emerged as the most suitable alternative to HDR for precise genome reconstitution after transgene insertion.

Optimising the specificity of genome editing

Although CRISPR has already revolutionised the way researchers are able to study gene function, an important limitation is so-called off-target effects where DNA cleavage occurs at unintended sites in the genome. For DNA cleavage to occur, there has to be sufficient complementarity between the gRNA and the genomic DNA sequence. However, Cas9 is tolerant of up to six mismatches to the target sequence,⁸⁹ which adds to the difficulty of finding unique target sequences within the human genome. The large size of the human genome raised the prospect of a twenty nucleotide spacer intended to bind a specific locus targeting thousands of additional genomic loci with sequence overlap, by chance. The computational prediction of gRNA activity and specificity, while initially limited by genome coverage and incomplete experimental data, has been greatly refined by experimental detection of off-target sites with an upper limit of sensitivity of $\sim 0.1\%$.⁹⁰⁻¹⁰⁰ Nonetheless, a substantial hurdle to overcome before considering downstream clinical applications of hiPSC is the importance of cellular context for gRNA activity. There is no computational method to accurately predict all off-target sites across the genome in every cellular context. The detection of unintended off-target effects is important, particularly in clinical applications, because even mutations restricted to a rare subset of clones carry the potential for oncogenic transformation or other unpredictable effects on cellular function. Below, we discuss the ways in which CRISPR-Cas target specificity can be optimised.

Improving specific genome editing by CRISPR nuclease engineering

The off-target effects revealed by genome-wide studies and restricted genome coverage of Cas9, constrained by its PAM requirement represent barriers to successful clinical translation of genome editing. To circumvent off-target effects, several engineered variants of SpCas9 that mitigate off-target editing have been developed by different laboratories. SpCas9-HF1,¹⁰¹ eSpCas9(1.1)⁹⁷ and HypaCas9 contain mutations that raise the threshold for the activation of Cas9 in the presence of a mismatch between the gRNA and the DNA target strand.¹⁰² HiFi Cas9 harbours a single point

mutation that reduces off-target effects without compromising on-target activity.¹⁰³ Other engineered variants, which are also functional in human non-PSC, are not constrained by an NGG (where N is an A, C, G or T nucleotide) PAM requirement, and so have much greater genome coverage. NG-Cas9,¹⁰⁴ and the naturally evolved Cas9 orthologue derived from *Streptococcus canis*, ScCas9¹⁰⁵ can target NG, GAA and GA-containing PAM sequences, respectively. SpG and, in particular SpRY are two other novel engineered variants of SpCas9 that offer much greater PAM flexibility, while retaining similar editing efficiency to WT Cas9.¹⁰⁶ The relaxed PAM requirement accounts for their greater off-target propensity, which was mitigated by engineering high-fidelity versions of the latter two nucleases.

The two catalytic domains of Cas9 which coordinately cut dsDNA have been engineered with mutations that induce distinct lesions in target DNA. The engineered Cas9 nickases harbour the HNH endonuclease mutations H840A and/or N863A, which nick one strand of genomic DNA.¹⁰⁷,¹⁰⁸ When combined with a pair of gRNAs targeting the sense and antisense DNA strands of the target region these Cas9 variants,¹⁰⁹ produce staggered DSBs, which improves the efficiency of precise HDR knock-in.¹¹⁰ Moreover, the frequency of off-target effects was markedly reduced in human non-PSC.¹¹¹ A simpler means of inducing staggered cuts in DNA is by using the type V-A CRISPR nuclease, Cas12a (formerly Cpf1).^{112, 113} In contrast to SpCas9, the target DNA sequence is cleaved as a 5-nucleotide staggered cut downstream of a T-rich PAM. Moreover, this CRISPR system lacks a tracrRNA, requiring only a single 42 nucleotide crRNA for DNA targeting. An additional advantage of Cas12a is its intrinsic RNase activity that can generate multiple gRNAs from a single concatemerized gRNA transcript, making Cas12a useful for multiplexed gene perturbation.¹¹⁴ An engineered version of Cas12a with greater PAM flexibility and gene editing efficiency, enAsCas12a should prove invaluable for precise HDR knock-in.⁴¹

Inducible versions of Cas9 improve gene editing specificity

Inducible forms of Cas9 controlled by an external stimulus minimise or prevent off-target activity as CRISPR activity is restricted to particular lineages or temporal windows.¹¹⁵ Early versions used cell-permeable small molecules to control Cas9 activity at the level of transcription. The ‘Tet’ switch derived from a bacterial antibiotic resistance operon is regulated by the antibiotics tetracycline (TET) and/or doxycycline (DOX) and is widely used in the control of gene expression in mammalian cells.¹¹⁶ An important feature of current third generation configurations of DOX-dependent Cas9 expression is the absence of ‘leaky’ expression of Cas9 when DOX is not present.¹³

Different approaches for integrating DOX-inducible Cas9 in the genome have advantages and drawbacks. Most often, DOX-inducible Cas9 has been knocked into the **adeno-associated virus integration site 1 (AAVSI)** safe harbour locus.¹¹⁷ The *AAVSI* locus lies within the **protein phosphatase 1 regulatory subunit 12C (PPP1R12C)** gene and remains transcriptionally active in hPSC and in their differentiated progeny.^{13, 117, 118} However, as differentiation proceeds the DOX responsiveness of Cas9 integrated at this site can attenuate, which hampers the use of inducible versions of CRISPR.¹³ Other safe harbour sites include two other widely used human loci, **C-C motif chemokine receptor 5 (CCR5)**^{119, 120} and *hROSA26*,¹²¹ and another locus, **citramalyl-CoA lyase (CLYBL)**,¹²² which was reported to be 10-fold more transcriptionally active than *AAVSI*. Given the limitation imposed by silencing on transgene expression in hPSC undergoing differentiation, comparing the fidelity of transcriptional activity from available safe harbour loci would be invaluable. Alternatively, multiple copies of *Cas9* can be randomly integrated throughout the genome without inducing DSBs, using transposon-based vectors.¹²³ Ideally, this should be followed by treatment with a transposase to excise the transgene in a ‘scarless’ manner and so avoid potentially disruptive genomic changes.¹²⁴

Post-translational induction of Cas9 requires fewer components and offers superior temporal control of CRISPR activity. In split Cas9 systems, activity is restored by subunit dimerization to form a functional Cas9 protein mediated by ligand-regulated intein splicing, or photoactivation.^{115, 125-127} Other forms of post-translational regulation of Cas9 exploit the coupling of Cas9 with protein domains that are photoswitchable,^{128, 129} temperature-dependent,¹²⁸ allosterically modulated by small molecules,¹³⁰ or which undergo ligand-regulated proteasomal degradation.^{131, 132} A modified split Cas12a can also be re-assembled to a functional form by chemical- or light-inducible heterodimerization of its N- and C-terminal halves.¹³³ Of these modalities of regulatory control, photoactivatable split Cas9 and ligand-dependent proteasomal degradation have been shown to be functional in hPSC.^{18, 127, 134} A potential difficulty with inducible forms of Cas9 and other nucleases is the possibility of pleiotropic effects of inducers (particularly small molecules) and undesired ‘leakiness’ of gene expression in the ‘off’ state.

Altering the delivery of CRISPR components facilitates precise genome editing

Plasmid transfection of CRISPR components favours extended expression of Cas9 protein and gRNA transcripts, which increases the likelihood of off-target effects.¹³⁵ Moreover, the dsDNA from which expression plasmids are constructed can cause cellular toxicity.⁵³ When inducible engineered variants of Cas9 or other CRISPR nucleases are required for conditional gene perturbation, plasmid-based delivery is still used because an external stimulus is needed to activate

CRISPR activity. Otherwise, to minimise off-target effects, Cas9 is usually delivered in a ribonucleoprotein (RNP) moiety containing either gRNA, or combined crRNA and tracrRNA in a complex with Cas9 protein.^{135, 136} Efficient delivery of HiFi Cas9¹⁰³ and type V CRISPR nucleases in this format has also been demonstrated.^{41, 137, 138}

The rapid onset and decay of CRISPR editing achieved through RNP delivery enabled efficient HDR editing, including homozygous knock-ins in hPSC and human non-PSC with minimal off-target effects and toxicity compared to plasmid vectors.^{52, 103, 135, 136, 139} Addition of anionic polymers such as poly-L-glutamic acid improve Cas9 RNP stability and improve knock-in efficiency in human primary T cells, without an increase in off-target indel formation compared to RNP alone.⁵² When RNP electroporation was combined with adeno-associated virus (AAV) transduction of the DNA template, there was a marked improvement in integration rates of large reporters.¹⁴⁰ Importantly, selection was not required which is important for clinical applications. However, the packaging capacity of AAV vectors is limited and prolonged transgene expression and the presence of viral proteins, raises concerns about off-target effects and immunogenicity when deployed *in vivo*.¹⁴¹ Newer methods utilise virus-like particles (VLP) lacking a viral genome to deliver the CRISPR cargo. Several VLP-based options, such as Nanoblades are highly efficient and functional in hiPSC and primary cells.¹⁴²⁻¹⁴⁵ They mostly depend on the retroviral polyprotein, Gag to package Cas9 and the gRNA within the VLP.^{146, 147} Synthetic VLPs offer substantial flexibility with respect to the the range of cell types that can be transfected by modifying their surface glycoproteins. VLP-based delivery also has low off-target effects compared to conventional DNA transfection. Moreover, template DNA for knock-in and multiple gRNA sequences for multiplexing can also be accommodated by VLPs. These VLP-based methods and viral delivery systems enhance the toolkit for therapeutic gene editing in the clinic.

Improving genome editing specificity through gRNA engineering

The sequence, length, stability and secondary structure of gRNA independently affects genome editing efficiency and strongly influences off-target cleavage rates.^{93, 148-153} Chemical modification of gRNA to prevent degradation by cellular RNases substantially increases the specificity of on-target dsDNA breaks.^{136, 154, 155} Rational gRNA secondary structure engineering is considered to improve on-target specificity by preventing unwanted activation of CRISPR nucleases at off-target sites.^{151, 153, 156} For example, addition of a hairpin structure onto the 5' end of the spacer region of gRNA increases the gene editing specificity of Cas9 and Cas12a in human non-PSC by several orders of magnitude.¹⁵⁶ Another modification of gRNA, an A-U flip in the stem-loop enhances gRNA stability by removing a putative terminator of Pol III transcription, which is commonly used

for heterologous gRNA expression.¹⁵⁷ An orthogonal version of an inducible CRISPR system, CRISPRoff incorporates photo-cleavable linker residues in gRNA that result in gRNA inactivation upon UV light exposure that has been tested in human non-PSC.¹⁵⁸ In this system, the level of genome editing was tunable by varying the time between transfection and UV exposure.

Reversible regulation of gene expression

Transient and reversible gene perturbation has informed the understanding of how distinct lineages can emerge from an initial pluripotent state,¹⁵⁹ and in modelling genetic risk variants for disease in cells differentiated from hPSC.^{160, 161} The tools for this purpose include synthetic transcriptional activators and repressors, and gene knockdown through mRNA perturbation, which in the CRISPR era, are based on nuclease-dead versions of Cas9 or its homologues. For stable activation of lineage-specific genetic programmes, these synthetic transcription factors activate gene expression at the endogenous locus in a sustained fashion, offer greater genome coverage and are cheaper than transient overexpression of natural transcription factors.¹⁵⁹ In this way, the genetic and epigenetic changes necessary to initiate distinct lineages can be defined, and the similarities and differences to endogenous pathways of differentiation understood.

CRISPR interference and activation

To circumvent the potentially irreversible and deleterious consequences of nuclease activity, CRISPR interference (CRISPRi) and activation (CRISPRa) can be used to perturb genes in hPSC reversibly, which allows greater flexibility in experimental design. In current implementations of CRISPRi, nuclease-dead versions of Cas9 (dCas9) or Cas12a (dCas12a) are fused to the Krüppel-associated box (KRAB) repression domain,¹⁶²⁻¹⁶⁴ to the mSin3 interaction domain (SID),¹⁶⁵ or to bipartite repressor domains leading to strong transcriptional interference.¹⁶⁶ The effectiveness of CRISPRi is greatest for sequences close to the transcription start site (TSS) due to a combination of nucleosome depletion, histone methylation and physical blockade of the early transcription complex,^{13, 167, 168} which limits potential off-target effects. TSS regions annotated in the FANTOM database have proved to be the most accurate for gRNA design.

Conversely, replacement of repressor domains with transcriptional activation domains (CRISPRa), commonly VP64, p65, and Rta (VPR) leads to enhanced gene expression. Like CRISPRi, CRISPRa is optimal when targeting gRNAs bind to genomic DNA sequences close to the TSS.^{156, 167, 169} Early versions required multiple gRNAs targeting the same promoter for robust transcriptional activation.¹⁷⁰ Second generation CRISPRa technologies, which combine multimeric transcription activator domains with dCas9 fusion greatly enhance target gene expression with minimal off-target effects.^{169, 171-175}

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3 Analogous dCas12a variants fused to transcriptional activators elicit strong and specific inducible or
4 constitutive transcriptional activation, and of these enAsCas12a performed best in a limited assay of
5 human genes.^{41, 133, 176, 177} As with conventional CRISPR-Cas, improved gRNA design rules that
6 incorporate gRNA secondary structure, gRNA protospacer length, chromatin accessibility, and
7 DNA sequence features in cell types of interest are also important for optimal CRISPRi/a
8 activity.^{150, 168, 176, 178} Despite the facile nature of CRISPRa in targeting protein-coding genes, high-
9 level gene expression might be offset by translational or post-translational mechanisms that limit
10 the levels of the corresponding protein.¹⁷⁹

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12 Early versions of CRISPRa targeting a pluripotency gene, octamer-binding transcription factor 4
13 (*OCT4*) in human fibroblasts, or to genes encoding the pancreatic transcription factors,
14 pancreas/duodenum homeobox protein 1, (*PDX1*) and NK6 homeobox 1 (*NKX6.1*) in hESC,
15 directed their fate to hiPSC and pancreatic cells, respectively.¹⁸⁰ Furthermore, hiPSC could be
16 differentiated into neurons by CRISPRa¹⁷² or by light-inducible, split CRISPRa transcriptional
17 activation of neurogenin2 (*NGN2*) or neurogenic differentiation factor 1 (*NEUROD1*).¹²⁷

18 ***CRISPR-mediated post-transcriptional control of gene expression***

19 This mode of gene perturbation is directed at the mRNA level, achieving comparable knockdown
20 efficiency to its predecessor, RNA interference (RNAi) but with far greater specificity.¹⁸¹ Its main
21 advantage over CRISPRi is the ability to reversibly target mRNA splice isoforms that has strong
22 clinical relevance. Leveraging patient-derived hiPSC to correct splicing defects for disease
23 modelling and cell therapy is worthwhile owing to the involvement of mis-splicing of RNA in up to
24 15% of genetic diseases.¹⁸² While Cas9 can be re-directed from dsDNA binding to RNA cleavage
25 or binding in a programmable fashion,¹⁸³ it has been surpassed by the RNA-guided RNA-targeting
26 Cas13 enzymes.^{184, 185} In particular, CasRx from *Ruminococcus flavefacien*¹⁸¹ outperforms other
27 family members in its efficiency of RNA knockdown; small size (~ 930 amino acids) making for
28 easier delivery; flexible mRNA targeting unconstrained by a PAM requirement; and superior
29 specificity in comparison to RNAi knockdown.¹⁸⁵ As a proof-of-principle, hPSC differentiated to
30 neurons, derived from patients with a neurodegenerative disease caused by abnormal splicing of the
31 microtubule associated protein tau (*MAPT*) gene,¹⁸⁶ were treated with the catalytically inactive,
32 dCasRx.¹⁸¹ The targeting of specific splice regulators in *MAPT* mRNA restored the balance of
33 splice isoforms, emphasising the potential for clinical application.

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57 **Base editors and Prime editors**

58 ***Base editors***

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A complementary approach to editing the genome makes use of engineered multicomponent proteins, which include CRISPR components to induce targeted single base changes in the genome. The broad repertoire of base editors engineered to optimise efficiency, target specificity, and base editing product purity have been comprehensively reviewed elsewhere^{187, 188} and are not discussed in detail here. In brief, there are two main classes of DNA base editors: cytosine base editors (CBEs) convert a C:G dinucleotide pair to A:T via a uridine intermediate, and adenine base editors (ABEs) induce a A:T to G:C substitution, via inosine (Fig. 4A, B). An ideal base editing tool would direct the desired alteration at single base resolution in a specified genomic location. Current versions of base editing are able to induce all four transition mutations (A-to-G, G-to-A, C-to-T, T-to-C) and a C-to-G transversion mutation,¹⁸⁹ within activity windows a few nucleotides in length for commonly used base editors, rather than at a single nucleotide. Base editing also operates in non-dividing cells that are refractory to DSB-stimulated HDR.

Base editing is functional in hiPSC, with the caveat that only a few studies have been published to date.^{190, 191} There are several reasons why base editing represents an important advance in genome editing for disease modelling and therapeutic use in hPSC. Base editors do not require DSBs to induce base edits, suggesting their potential to avoid any deleterious genomic effects associated with CRISPR editing in hPSC. The potential of base editing to treat monogenic diseases caused by single base mutations was demonstrated in the premature ageing disease, Hutchinson-Gilford progeria syndrome (HGPS). An adenine base editor reversed pathogenic C > T mutations in the lamin A (*LMNA*) gene in patient fibroblasts *in vitro*¹⁹². In addition, exon skipping is a promising treatment for several monogenic diseases, including Huntington's disease¹⁹³ and DMD.¹⁹⁴ In principle, in DMD, skipping exons 1 and 2 would cover over 80% of all DMD mutations.¹⁹⁵ Whereas CasRx has only a transient effect on exon splicing,¹⁸¹ base editors can alter the splicing of mRNA by simultaneously and permanently disrupting multiple highly conserved splice acceptor sequences in genomic DNA.¹⁹⁶ The resulting exon skipping can encompass multiple exons in a single transcript, which is often necessary for therapeutic applications.¹⁹³ In principle, the mitochondrial genome should also now be amenable to genome editing in hPSC. Mitochondrial DNA (mtDNA) mutations feature neurological, muscular and metabolic manifestations.¹⁹⁷ Until recently mtDNA has been inaccessible to genome editing, because the electrochemical gradient across the inner mitochondrial membrane is a barrier to nucleic acids. This problem was recently overcome and precise single base edits to mitochondrial genes in human non-PSC are now possible using a fusion protein incorporating a novel cytidine deaminase.¹⁹⁸

Prime editing

Prime editing represents another milestone in genome editing that is capable of inducing targeted insertions, deletions, knock-in epitope tag sequences, Cre recombinase loxP sites, and all possible base-to-base conversions including the transversion mutations, A-to-C, A-to-T, C-to-A, T-to-A, G-to-C, G-to-T, C-to-G, T-to-G (Fig. 4C, D).¹⁹⁹ Furthermore, the desired mutations can be induced at single base resolution rather than in a window several bases long. Therefore, prime editing has greater flexibility than base editing in the range of editing choices that can be made. The core molecular components consist of a Cas9 nickase (H840A HNH mutant), reverse transcriptase (RT) and a prime editing gRNA (pegRNA) that targets the genomic sequence of interest and encodes the desired edit. The editing function is localised to the 3' sequence of the pegRNA, which contains a priming sequence that binds to the 3' end of the nicked DNA strand, and a reverse transcriptase template sequence containing the desired edit.

Prime editing is still in its infancy, but has already been successfully tested in adult human intestinal and liver stem cells,²⁰⁰ human embryonic stem cells (<https://www.biorxiv.org/content/10.1101/2021.04.12.439533v1.full>) and in hiPSC.¹⁹¹ Relevant to clinical translation, and consistent with findings in human non-PSC lines^{48, 201, 202} in these adult stem cells, prime editing outperformed Cas9-mediated HDR for the generation of precise targeted deletions and in the frequency of genome-wide off target effects.²⁰⁰ Moreover, in both hiPSC and in adult stem cells, prime editing efficiency improved with shorter priming sequences.^{191, 200}

A bottleneck in the widespread adoption of prime editing is the difficulty of synthesizing and cloning pegRNAs. Moreover, prime editing is currently limited to plasmid-based applications as it requires prolonged expression of Cas9n-RT which is unlikely to be achieved by delivery in an RNP. However, these issues might be solved by the advent of VLP-based systems.^{142, 143} The frequency of an NGG PAM on either DNA strand, averaging every eight nucleotides does not constrain the genomic coverage of prime editing, because edits can extend over 30 base pairs from the PAM. However, if no suitable NGG PAM were present in the target region of interest, the Cas9n HNH mutant could in theory be replaced with a Cas12a nickase variant or an engineered SpCas9 nickase with an expanded PAM repertoire.

Scaling up gene perturbation in CRISPR screens

The sequencing of the human genome poses a new challenge of determining the functions of identified genes, and their regulatory elements. Developing models of gene function for thousands of genes through genetic perturbations of single genes in model organisms is time consuming and laborious. Pooled forward genetic screens employ a massively parallel design to perturb genes at up

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to a genome-wide scale, with the aim of uncovering their role in phenotypes such as cell viability, drug resistance or marker expression.^{100, 203} CRISPR screens, with their greater consistency and effectiveness outperform earlier modalities of forward genetic screens, with little overlap of gene sets.²⁰⁴⁻²⁰⁶ Their utility is greatest in identifying genes with cell-autonomous effects, whereas lower-throughput arrayed screens, not discussed here, are necessary to detect non-autonomous gene functions.

Most often, cells stably expressing a CRISPR nuclease (commonly Cas9 or Cas12a) are transfected in bulk with a library of gRNAs cloned into a suitable delivery vehicle, usually a lentiviral vector (Fig. 5A). gRNA knockout libraries with genome-wide coverage are well-developed and are available through Addgene.²⁰⁴ Custom libraries to target sets of genes can also be generated and are facilitated by freely available gRNA design software.²⁰⁴ Transfection conditions are adjusted so that typically each cell stably integrates a single gRNA construct. Positive and negative selection screens are used to identify gRNAs that are enriched (due to cell proliferation) or depleted (due to cell death), respectively, pointing toward the function of the corresponding targeted gene (Fig. 5A). Potential off-target effects of the gRNA need to be carefully considered as multiple dsDNA breaks can lead to apoptosis and dropout of cells, which confounds viability screens. Furthermore, copy number amplified regions in the genome will also be susceptible to being erroneously called through the same mechanism.

CRISPRi or CRISPRa screens can be more informative than CRISPR knockout when screening regulatory elements or non-coding RNA, when the effect of a small indel could have little discernible effect on function, and to screen for gain-of-function phenotypes.^{204, 207} They are also less prone to off-target effects than CRISPR knockout screens, because CRISPR activity is confined within the narrow region of the TSS.¹⁶⁷ A further advantage is that the effect of combinatorial gene perturbations, using CRISPRi screens in iPSC in particular, on a phenotype of interest can be tested without triggering an apoptotic response.²⁰⁸ Confounding effects can nevertheless arise when using these forms of genetic screening, if a bi-directional promoter is targeted resulting in the silencing or activation of more than one gene. As with CRISPR knockout, off-the-shelf genome-scale CRISPRi and CRISPRa gRNA libraries are also available on Addgene.^{168, 204}

In pooled CRISPR screens, the phenotypic consequences are ‘averaged’ across the set of gRNAs targeting an individual gene without insight into how the gene perturbation leads to the phenotypic change. Furthermore, in a conventional negative selection screen, the depletion of clones is

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3 measured by the relative abundance of the gRNA. gRNAs effective in introducing mutations that
4 affect cell viability will be depleted out of the cell pool. In practice however, the same guide might
5 generate distinct indels in different cells, resulting in loss-of-function (LOF) in some cells, whereas
6 other cells acquire hypomorphic or hypermorphic mutations (Fig. 5B). To overcome this problem,
7 random barcodes detectable by high-throughput sequencing linked to each gRNA can be used,
8 which enables lineage-tracing of single clones and improves screen accuracy and resolution (Fig.
9 5C, D).²⁰⁹⁻²¹¹ The mechanism of phenotypic change has also been addressed using single cell
10 transcriptome sequencing (scRNA-seq) to classify cell states in response to genetic perturbation.²¹²⁻
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20 Second generation pooled CRISPR knockout screens, thus far conducted in human non-PSC lines,
21 improve on the efficiency of prior screens by using engineered Cas12a variants that can process
22 multiple gRNAs from a single transcript.^{216, 217} Advantages of this approach are that fewer cells are
23 needed, making organoid cultures and primary cells where cell numbers are limiting, targetable.
24 Moreover, multiplexed gene knockout in most cells is feasible. Multiplexing allows the
25 simultaneous targeting of gene paralogs where knockout of a single gene is insufficient to reveal a
26 phenotype. Importantly, suitable controls were used to separate out the non-specific effects of
27 multiple dsDNA breaks on cell viability. These controls are especially important when hPSC are the
28 chosen cell type in which to undertake the screen.

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31 High-throughput CRISPR loss-of-function screens can address important questions unique to the
32 biology of hPSC, for example the networks of genes that regulate their growth and pluripotency.^{18,}
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210, 218, 219 hPSC are an invaluable resource to investigate the acquisition of normal and disease
phenotypes of primary human cells that are post-mitotic or difficult to obtain in sufficient
quantities. To this end, an hiPSC neuronal differentiation model was used in pooled CRISPRi¹³⁴
and CRISPRa²²⁰ screens to identify the genetic regulators of neuronal differentiation, morphology
and survival, whereas a CRISPR knockout screen identified an important molecular pathway of
neurodegeneration.²²¹

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Conclusions and future directions

In this overview we highlight CRISPR/Cas technologies that are functional in hPSC. With these
tools it is possible to address diverse questions pertaining to the biology of these cells and tailor
their use for clinical applications. Often, the goal is to determine the function of a gene(s) in a
healthy or diseased state, or in the context of a drug treatment. When the intention is to study the

genetic networks that regulate pluripotency, lineage commitment, or differentiation the choice of CRISPR perturbation will be influenced by the purpose of the edit. For gene knockout, NHEJ-based indel generation is the most straightforward approach. Conditional gene targeting is more time-consuming and complex, relies on homology- or micro-homology-dependent repair for precise gene knock-in and can be hampered by transgene silencing. In the latter context, localisation of the CRISPR effectors to the nucleus with an ssDNA repair template improves the efficiency of obtaining correctly targeted clones. Likewise, homology-dependent methods are usually used to track cell lineages, or tag a protein. Newer, and highly efficient methods based on NHEJ repair broaden the options for precise knock-in.

In future, conditional targeting in hPSC could benefit from the added flexibility of stimulus-regulated gRNA molecules incorporating ligand-activated riboswitches, which are RNA molecules mostly derived from bacteria.²²² A fundamental question in hPSC biology relates to their propensity to differentiate along particular lineages and how this is linked to specific PSC cell states, defined on the basis of molecular and cellular differences.²²³ These include their miRNA profiles²²⁴ that can be detected using synthetic constructs expressed in hPSC, leading to activation or repression of CRISPR/Cas9 genome editing.²²⁵ Once optimised, novel CRISPR approaches like this should, in future, help to dissect the regulation of distinct pluripotent cell states.

The synergistic effect of combining hPSC differentiation protocols with CRISPR/Cas technology has strong potential to drive major advances in disease modelling and regenerative medicine (Table 1). In parallel, the search for disease-relevant cellular phenotypes that can be used to test the effects of drugs or CRISPR mutations, is an important area for development. For disease modelling, when single nucleotide changes are sought, for instance when an allelic series of isogenic lines to model splicing abnormalities is required, base editors are ideal.²²⁶ For small, precise insertions and deletions the choice lies between prime editing and homology-dependent repair, with the latter modality better established than the former. Following gene editing, assays for genome integrity, for example karyotyping or a SNP array, should be performed.

Cell therapies lag behind disease modelling in hiPSC translational applications, yet there is a strong unmet need for such treatments. **In haematological malignancies, for example**, autologous donor haematopoietic stem cells (HSC) differentiated from patient-generated hiPSC would avoid immunological rejection. However, the efficiency of HSC differentiation is low,²²⁷ making the case for CRISPR screens to identify the roadblocks to HSC differentiation and the genetic drivers of

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3 differentiation. The utility of CRISPR screening as a step towards eventual cell therapy was
4 recently demonstrated in a non-PSC line for the treatment of another disease. In Type 1 diabetes
5 mellitus, a barrier to autologous iPSC-derived insulin-secreting beta cell replacement is
6 autoimmune attack of transplanted cells.²²⁸ A CRISPR screen identified a key gene, whose
7 suppression by a re-purposed FDA approved drug decreased the vulnerability of transplanted beta
8 cells to autoimmunity.²²⁹ In haematologic and solid cancers, the converse situation of immune
9 evasion by the cancer reduces the efficacy of T-cell based treatments.²³⁰ A potential solution to this
10 problem is suggested by a proof-of-principle study in which hiPSC-derived natural killer (NK) cells
11 expressing a cleavage-resistant variant of the immunoglobulin receptor were endowed with
12 enhanced anti-tumour activity.²³¹ Moreover, this novel experimental cell-based treatment can be
13 delivered *in vivo* by infusion, which should accelerate translation to the clinic.
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23 The combination of iPSC and CRISPR technologies has great therapeutic potential in accessible
24 tissues with deficient or defective adult stem cells, and when a single genomic edit is sufficient to
25 restore normal function in differentiated progeny. The haematopoietic system is once more an
26 exemplar, because of the relative ease of replacing diseased cells in this organ. Clinical protocols
27 for the creation of space in the bone marrow for *ex vivo* engineered immune cells in advance of their
28 infusion could be borrowed or adapted from HSC transplant, or gene therapy for severe combined
29 immunodeficiency (SCID), regimens.²³² In the monogenic blistering skin disease, Dystrophic
30 Epidermolysis Bullosa, CRISPR correction of patient iPSC followed by differentiation and skin
31 grafting in mice demonstrate the strong clinical potential of autologous skin replacement.²³³ The
32 latter diseases provide examples of accessible tissue or organ contexts into which new cells can
33 integrate and continue to function. For other organs such as the central nervous system, cell
34 replacement therapies will prove substantially harder to deliver, with the possible exception of the
35 retina.²³⁴ Separately, the functional immaturity of many differentiated cell types is a major hurdle
36 for the implementation of cellular therapies.²³⁵
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49 An important consideration in cell-based treatments is the safety of transplanted cells, which can
50 form teratomas or other tumour types.^{236, 237} For this reason, hiPSC lines have been engineered with
51 small molecule activated ‘safety switches’ which incorporate endogenous effectors of apoptosis in
52 the event of adverse outcomes of cell transplants.²³⁸ Ideally, monitoring the safety of cell-based
53 treatments *in vivo* should rely on clinical examination or minimally invasive laboratory tests, which
54 is readily achieved for skin and the haematopoietic system. Thanks to the ever more refined and
55 expanding CRISPR toolkit, improvements in targeting efficiency and editing precision look set to
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continue at pace. To deliver the considerable promise of hPSC technologies for patients the stem cell community in partnership with clinicians should begin to address future challenges. These include delivery, integration and monitoring of PSC-based therapies in humans given that precise correction of genetic defects in hPSC looks increasingly tractable.

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For Peer Review

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Table 1. *Ex vivo* gene editing of ESC and iPSC and potential translational applications

<i>CRISPR technology</i>	<i>Example disease</i>	<i>CRISPR intervention</i>	<i>PSC technology</i>	<i>Potential translational application</i>	<i>Reference</i>
<i>NHEJ knockout</i>	HIV	Knock-out of <i>CCR5</i>	Macrophage differentiation of patient iPSC	Transplantation of autologous HIV resistant macrophages	[1]
<i>NHEJ gene therapy</i>	Duchenne Muscular Dystrophy	<i>DMD</i> exon 45 skipping using dual gRNAs targeting SA and SD	Skeletal muscle cell differentiation of patient iPSC	Transplantation of autologous Dystrophin expressing skeletal muscle cells	[2]
<i>MMEJ knockout</i>	DYSF deficient muscular dystrophies	Loss-of-function mutation in <i>DYSF</i>	Myogenic differentiation of ESC/iPSC	Generation of disease models with isogenic controls for functional analysis and therapy testing	[3]
<i>MMEJ gain of function</i>	X-linked Protoporphyrin	Gain-of-function mutation in <i>ALAS2</i>	Erythroid differentiation of ESC/iPSC	Generation of disease models with isogenic controls for functional analysis and therapy testing	[4]
<i>HDR correction</i>	Amyotrophic lateral sclerosis	Excision of the <i>c9orf72</i> repeat expansion	Astrocyte differentiation of patient iPSC	Disease modelling and potential transplantation of autologous corrected astrocytes	[5]
<i>HDR knock-in</i>	Duchenne Muscular Dystrophy	Knock-in of exon 44 of <i>DMD</i>	Skeletal muscle cell differentiation of patient iPSC	Transplantation of autologous Dystrophin expressing skeletal muscle cells	[6]
<i>HDR gene therapy</i>	Haemophilia B	Knock-in of WT copy of <i>F9</i>	Hepatocyte differentiation of patient iPSC	Transplantation of autologous factor IX producing hepatocytes	[7]
<i>Base Editors</i>	Spinal muscular atrophy	Conversion of exonic splicing silencers by single base editing of <i>SMN2</i> exon 7	Motor neuron differentiation of patient iPSC	Transplantation of autologous <i>SMI-like</i> expressing motor neurons	[8]
<i>CRISPR screen</i>	Diabetes Mellitus	Genome wide CRISPR screen that showed knock-out of <i>RNLS</i> reduces autoimmune vulnerability	Beta-cell differentiation of ESC/iPSC	Identification of genes associated with autoimmune resistance in pancreatic beta-cells	[9]

Legend: *ALAS2*: 5'-Aminolevulinate Synthase 2, *CCR5*: C-C Motif Chemokine Receptor 5, CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats, *DMD*: Dystrophin, *DYSF*: Dysferlin, ESC: Embryonic Stem cell, *F9*: coagulation factor IX, (i)PSC: (induced) Pluripotent Stem Cell, HDR: Homology Directed Repair, HIV: Human Immunodeficiency Virus, MMEJ: Microhomology mediated end-joining, NHEJ: Non-homologous End-joining, *RNLS*: Renalase, SA: splice acceptor, SD: splice donor, *SMN1*: survival of motor neuron 1, *SMN2*: survival of motor neuron 2 .

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For Peer Review

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FIGURE LEGENDS

Figure 1. DNA repair pathways after CRISPR induced DNA double strand breaks. **A.** The three main pathways involved in DNA repair after a gRNA directed (white), Cas9 (deep blue) induced double strand break (DSB) are homology directed repair (HDR), non-homologous end joining (NHEJ) and homology-mediated end joining (HMEJ). HDR uses homologous regions of DNA, such as a sister allele as a template for DNA synthesis and correct DNA repair. DNA ends are resected to allow invasion of the homologous DNA from the sister allele that acts as a template for repair. NHEJ requires processing of the DNA ends before the DNA is ligated to repair the DSB. This process is error-prone and frequently results in insertions or deletions of base pairs. In HMEJ, break ends are resected to reveal intrinsic regions of homology that are found on both sides of the DSB (light blue). Once revealed, these regions can anneal and synthesis and ligation can proceed to repair the DSB. This mechanism trims off the sequence between the homology regions (red). When the homology regions are short (2 – 25 bp), this mechanism is termed microhomology mediated end joining (MMEJ). **B.** HDR mediated knock-in can be achieved by supplying a DNA template with homology arms (orange and purple) flanking the knock-in sequence (green). In NHEJ knock-in, the target sequence (green) is ligated with the DNA ends and thus integrated into the genome. HMEJ knock-in requires a DNA template with homology arms (blue) that are usually shorter than those for HDR knock-in. The homology arms on the template anneals with the homology regions in the DNA and after DNA synthesis and ligation the target (green) is integrated.

Figure 2. Methods for improving the efficiency of precise knock-in by HDR. **A, B.** Double selection strategy to enrich for correctly targeted hPSCs without unintended edits at the targeted locus. A membrane-bound, surface epitope is used for both positive and negative selection of cells by magnetic activated cell sorting (MACS). **A.** In the first round of editing, the selection marker and a fluorescent reporter in tandem with the corrected sequence (1st donor) is inserted. MACS positive selection followed by single cell cloning, aided by fluorescence microscopy for the reporter is used to identify mono-allelically (pink) and bi-allelically (red) integrated clones. In the second round of HDR, the reporter (1st donor) is excised and replaced with the second donor. MACS is used for negative selection to isolate cells with the corrected sequence and lacking the reporter. **B.** The intermediate product (1st Product) containing the desired edit (green), a fluorescent reporter (pink) and a cell membrane epitope (orange) is inserted in the 1st round of HDR. In the 2nd round of HDR, the reporter and cell membrane epitope are excised. **C.** When ‘scarless’ editing is used for protein tagging, in this example at the C-terminus, positive selection for a second reporter (green) is used.

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3 Distinguishing between correct and incorrect (or random) integration of the second reporter can be
4 difficult. Appending shRNA sequences (dark blue) targeting expression of the inserted reporter to
5 the 5' and 3' ends of the donor template allows for the identification of cells with random
6 integration. In these cells, the shRNA sequence is integrated and the expression of the reporter will
7 be silenced resulting in absence of the fluorescence signal.
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13 **Figure 3** Alternatives to HDR for precise transgene integration. **A.** MMEJ using the PITCh system,
14 in this example to integrate a transgene upstream of a stop codon (STOP) to facilitate protein
15 tagging. PITCh uses one gene-specific gRNA, with ~20 bp microhomologies. This results in highly
16 accurate reconstructions of the junctions between the transgene and genomic DNA. In constructing
17 the PITCh vector, common PITCh-gRNA target sites for a second gRNA are introduced upstream
18 and downstream of the 5' and 3' microhomologies, respectively. **B.** CRISPaint tags a protein of
19 interest at its N- or C-terminal using NHEJ. A universal donor plasmid is used that contains a cut
20 site with a stretch of six guanine nucleotides, the sequence encoding a fluorescent reporter tag and a
21 puromycin selection cassette. A gRNA in a separate 'target selector' plasmid targets the genomic
22 sequence of interest. The donor can be cut in all possible reading frames using an appropriate
23 'frame selector' gRNA expressed from a second plasmid. The choice of which frame selector to use
24 depends on two considerations. First, the tag sequences should lie in frame with the gene of interest.
25 Second, the three nucleotides upstream of the chosen PAM in the 'frame selector' and the genomic
26 sequence should differ by at least two nucleotides. Otherwise, the efficiency of integration is poor
27 because of re-cutting of the fusion gene instigated by the target selector gRNA. Cas9 can be co-
28 delivered from the target selector plasmid or cells already transfected with Cas9 can be used.
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43 **Figure 4** Mechanism of CRISPR base editing and prime editing. **A.** The base editor is composed of
44 a Cas9 H840A nickase (Cas9n) or a dead Cas9 (not depicted) that is fused to a single-strand DNA
45 specific deaminase, in this example a cytidine deaminase. Binding of the guide RNA (gRNA)
46 creates an R-loop that exposes a small sequence of single stranded DNA (ssDNA) to the ssDNA-
47 specific cytidine deaminase, which targets cytosine (red) and Cas9n induces a nick (arrowhead) in
48 the opposite strand. **B.** Cytidine deaminase converts cytosine to uracil. Nicking of the opposite
49 strand induces DNA repair using the edited base as a template. DNA replication then completes the
50 process of C-to-T conversion. **C.** Prime editing alters a pre-defined genomic sequence without
51 generating double-stranded DNA breaks and does not require a donor template. Cas9n is directed to
52 its genomic target by the 5' part of the pegRNA containing the spacer. Upon binding, the non-
53 template strand (top strand) is nicked and the 3' end of the nicked DNA strand is released by the
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Cas9 nickase. The released 3' end hybridizes with the 3' end of the pegRNA and becomes a substrate for RT. As a result, the desired mutation (located in the 3' end of the pegRNA) is introduced into the non-template strand of the genomic DNA by RT. **D.** Upon completion, the newly transcribed strand is released and competes with the unedited 5' end to re-anneal with its original hybridization partner (equilibration). Although the unedited 5' end is perfectly complementary to the unedited strand and therefore favored, 5' flaps are the preferred substrate for structure-specific endonucleases. As a result, the Prime Edited 3' end is preferentially incorporated, creating heteroduplex DNA containing one edited strand and one unedited strand. The endogenous DNA repair machinery then resolves the heteroduplex by copying the information in the edited strand to the complementary strand via a mechanism that is currently poorly understood.

Figure 5 Pooled CRISPR screen designs. **A.** Pooled CRISPR screen experimental workflow. Each gRNA targets a specific gene in the genome, and typically multiple gRNAs are used to target a single gene. Each gRNA in a lentiviral particle is distinct and is represented by a different colour. Each cell or set of cells harbours a mutation in a different gene. When selection is applied, some mutations will result in increased survival/proliferation (red, yellow and green gRNAs), while others will cause cell death or decreased proliferation (blue, orange and purple gRNAs). Genomic DNA extraction and next generation sequencing (NGS) enables the screening of the cell pool for gRNAs that are enriched (positive selection screen) or depleted (negative selection screen). This indicates a positive or negative effect of the gene knock-out on cell survival/proliferation, respectively. **B.** Conventional negative selection screens lack the resolution to distinguish gRNAs causing gene knock-out from other effects. In this example, gene A (red) is important for cell survival, while gene B (green) does not affect survival. However, only correct bi-allelic mutations in gene A result in decreased cell survival. Cells that have mono-allelic mutations or non-loss-of-function mutations survive and contribute to the gRNAs found in the cell pool. When screening for depleted gRNAs (negative screen), the relative abundance of gRNAs targeting gene A gives the false impression that gene A does not affect cell survival. This is an important limitation of this type of screen. **C.** Principle of barcoded gRNAs in pooled CRISPR dropout screens. Cells can be distinguished based on their unique gRNA barcode, which is used to assign a unique molecular index (UMI) to individual cells, or clones. After transfection, cells undergo strict single cell cloning to create pools of cells derived from a single clone. Under selection, mutant genotypes can result in complete gRNA depletion because of cell death (Gene A, UMI1 and UMI2), no depletion in the event of a non-LOF mutation (Gene A, UMI 3), or partial depletion (Gene B, UMI 4 and UMI5). Each editing outcome at single clone level is associated with a UMI that can be detected by NGS. UMIs thus

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3 provide biological replicates within the same experiment, allowing the different phenotypic
4 outcomes to be distinguished, resolve cellular heterogeneity, and identify outliers that would
5 otherwise confound results. **D.** Example of a positive selection screen using CRISPR-UMI to
6 identify genes that influence re-programming of mouse embryonic fibroblasts (MEFs) to iPSCs.
7
8 High-throughput sequencing is used to generate two key metrics that reflect the clonal distribution
9 pattern of iPSCs after transfection with the gRNA library. The number of distinct UMIs for a gRNA
10 reflect the number of independent iPSC colonies, whereas the read count for each UMI reflects the
11 growth kinetics of individual iPSC colonies. A larger read count implies a larger colony.
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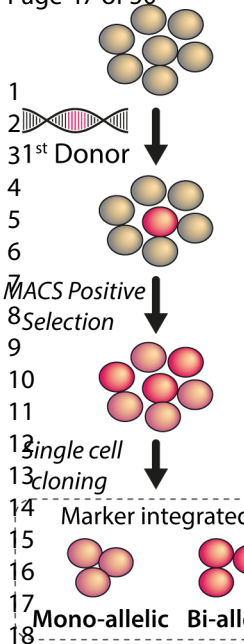
For Peer Review

A**Double strand DNA break**

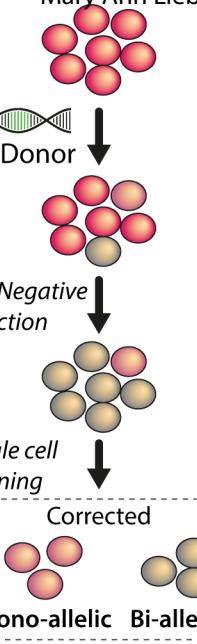
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**HDR****NHEJ****HMEJ***Resection**End processing**Resection**Synthesis**Ligation**Annealing**Synthesis / Ligation**Correct DNA repair**Indels**Variable size deletions***B****HDR knock-in****NHEJ knock-in****HMEJ knock-in***DNA template**DNA template**DNA template*

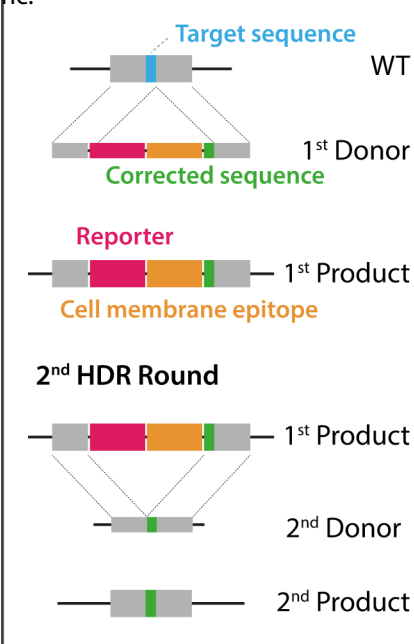
A 1st HDR Round



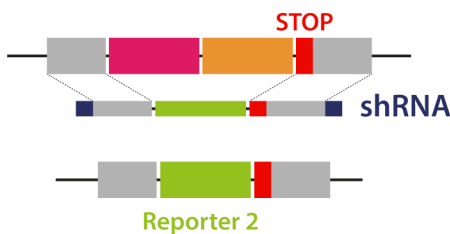
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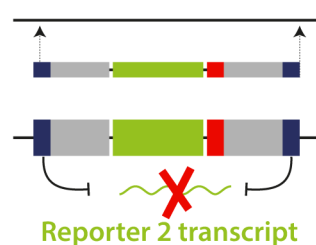
C 1st HDR Round

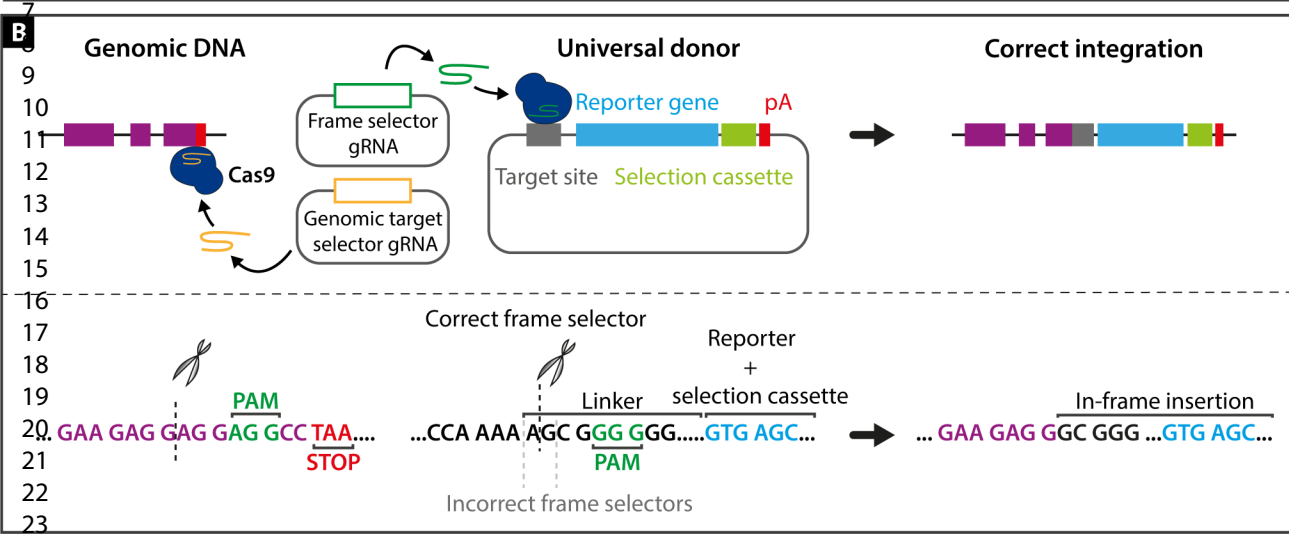
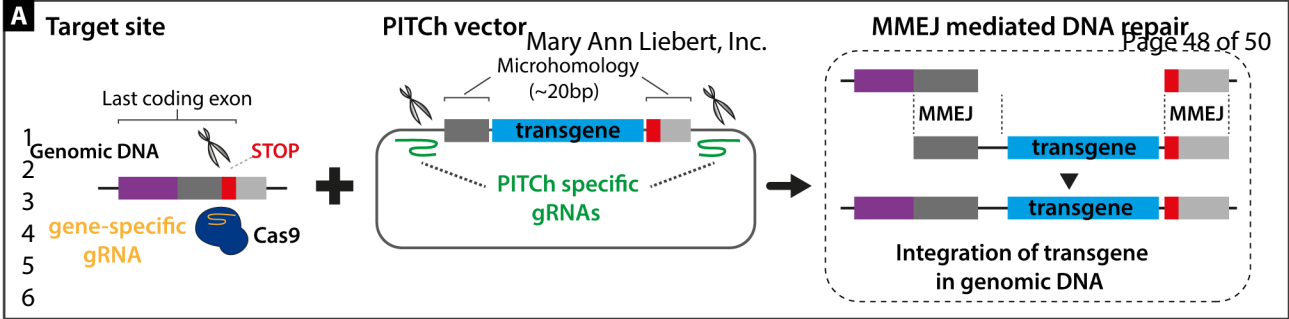


Correct integration

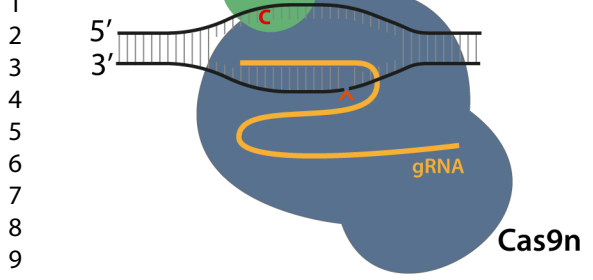


Random integration

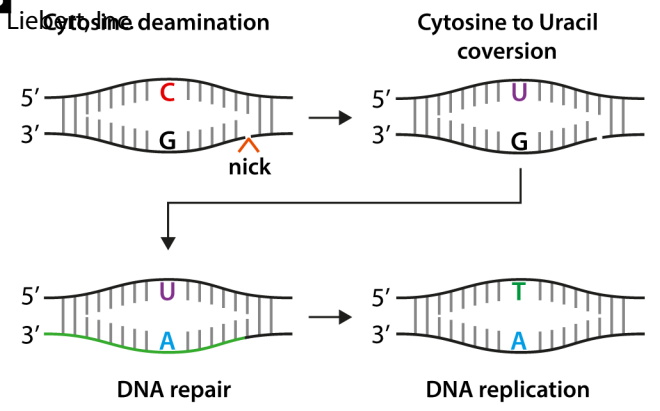




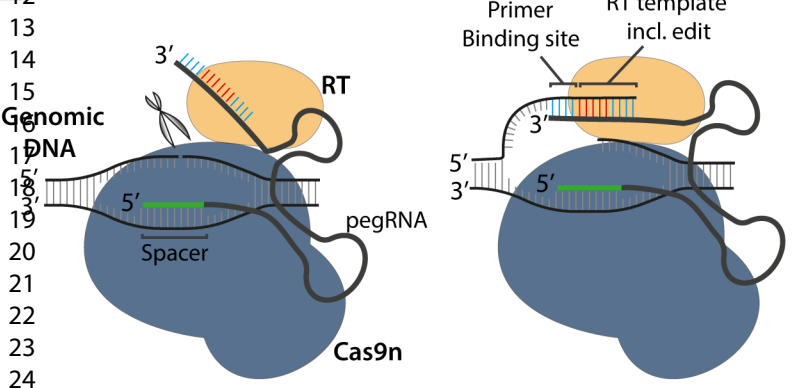
A
Exposure of ssDNA
in R-loop



B
Cytosine deamination



C
Genomic
DNA



D

