Genome Editing Applications for Disease Modeling and Cell Therapy

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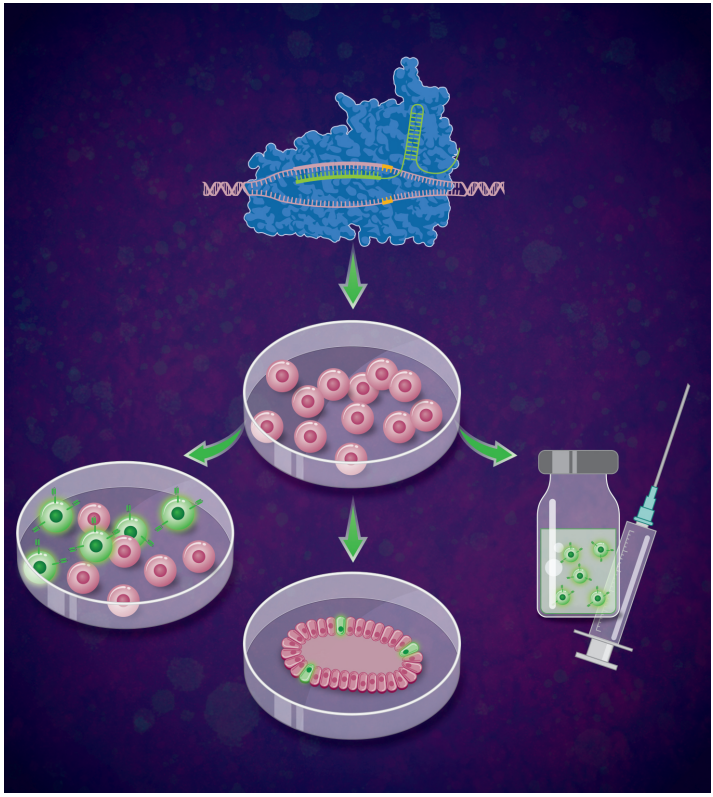
INTRODUCTION

DNA is the blueprint of life that encodes RNA and protein, as well as instructions for generation of the many different cell types that assemble into an organism. Genome editing, or the purposeful alteration of an organism's DNA sequence, has been a long-standing goal for scientists. Efforts to carry out genome editing can be categorized into three main subtypes:

1. Generation of non-specific mutations at non-specific loci. This is typically achieved by phenotype-based selection of naturally occurring genetic variants or radiation- or chemical-induced random mutagenesis.
2. Insertion of specific genetic sequences into non-specific loci. The first genetically modified organism was generated through injection of preimplantation mouse blastocysts with Simian virus 40 (SV40) (***1***). This method allows for transgene expression from random sites in the genome where the virus DNA integrates but is incapable of precisely 'editing' a specific gene sequence.
3. Precise genome editing at specific loci. Precise genome editing has become possible due to the discovery of naturally evolved nuclease proteins, and subsequent protein engineering efforts to harness and transform their power for a variety of applications. Precise genome editing primarily relies on the controlled induction of DNA double-strand breaks (DSBs), and their subsequent repair by endogenous DNA repair mechanisms.

This book introduces the reader to genome editing technologies, with a specific focus on the CRISPR-Cas system and how it is being successfully applied in human cells for basic and translational research. We discuss some of the major challenges that remain in the field and touch on strategies to overcome these problems. Finally, we speculate on future directions for this exciting and fast-paced field.



CRISPR-Cas9 Technology and Application: Genome editing with CRISPR-Cas systems has revolutionized biomedical research. CRISPR-Cas9 functions as a complex of specialized RNA molecules and protein enzymes to precisely edit genomic DNA at specific sequences (top). This molecular tool can be applied in vitro and in vivo to modify the function of various cell types such as immune cells (left culture dish), to engineer relevant disease models in complex culture systems such as organoids (center culture dish), or to generate novel cell-based therapeutics to be used in the clinical setting (right dish).

A BRIEF HISTORY OF PRECISE GENOME EDITING TECHNOLOGIES

Restriction enzymes are one of the first discovered classes of proteins that induce site-specific DSBs, and their application spawned a new age of *in vitro* recombinant DNA technology. These enzymes, generally of bacterial origin, recognize and bind to a suite of stereotypical short DNA sequences termed 'restriction sites' and cleave nearby DNA. While restriction enzymes have long been used in the manipulation of DNA plasmids for molecular cloning purposes, the frequent occurrence of their recognition sequences (typically 6–8 bp) impedes their application as sequence-specific genome editing tools in the context of an entire genome.

Designer nucleases were developed to increase the sequence specificity of genome editing. These meticulously engineered proteins, including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), consist of non-specific nucleases fused to sequence-specific DNA binding domains. The DNA binding domains provide target specificity for nuclease-dependent generation of DSBs in the neighboring sequence. Zinc finger domains recognize trinucleotide DNA sequences, enabling scientists to combine and fuse different moieties to the cleavage domain of non-specific DNA endonucleases, such as Fok I (*2*), for targeted DSB generation. TALENs were later added to the genome editing toolbox, providing an additional level of sequence specificity via single nucleotide recognition (*3*). ZFNs and TALENs were engineered to bind specific DNA sequences (typically 12–20 bp) to mediate precise DSB generation and subsequent genome editing. However, their complicated design and relatively low efficiency severely limit the broad application of ZFNs and TALENs in genome editing experiments.

The rapid discovery and repurposing of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) greatly advanced the field of precise genome editing. The CRISPR system involves RNA 'guides' that instruct site-specific binding of CRISPR-associated (Cas) proteins to mediate DNA or RNA cleavage. One of the first and most widely adopted CRISPR-associated variants, Cas9, once paired with a guide RNA (gRNA),

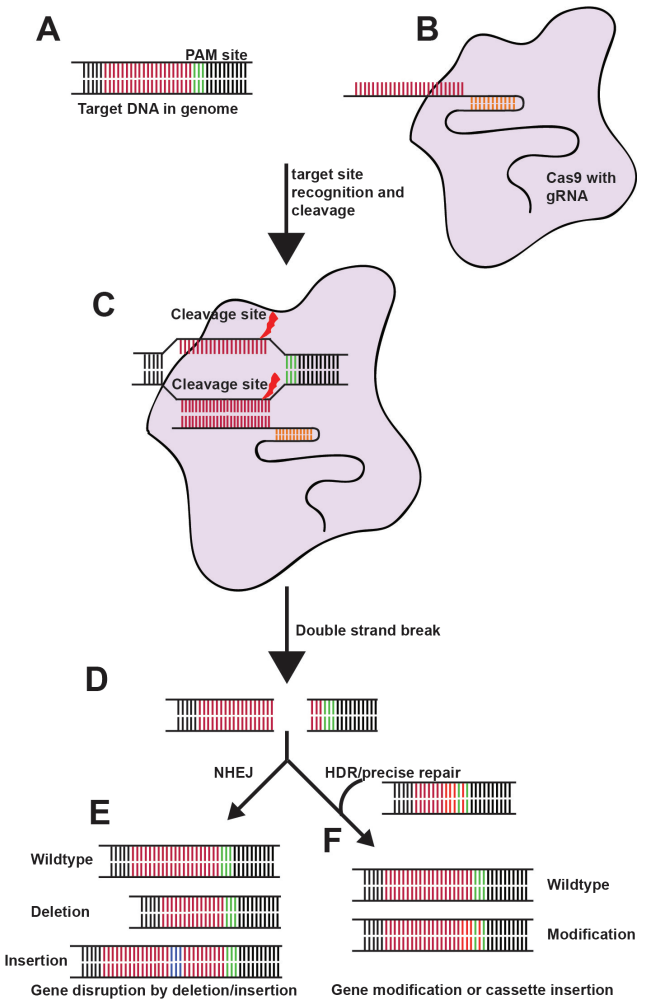


Figure 1: Targeted DNA editing by CRISPR/Cas9 system.

Target DNA sequence (A) and Cas9 with corresponding guide RNA (gRNA) (B). The CRISPR-Cas9 genome editing system consists of ~100 nucleotide guide RNA (gRNA) in complex with Cas9 protein. Cas9 searches the genome for protospacer adjacent motif (PAM) sites immediately downstream of a sequence complementary to the ~20 nt protospacer/crRNA sequence within the gRNA. Cas9 will then introduce a double-strand breaks (DSB) (C and D). Cas9-induced DSBs are then repaired by either the non-homologous end joining (NHEJ) (E) or homology-directed repair (HDR) pathway, which defines editing outcome (F). Errors introduced by NHEJ can generate a variety of insertions and deletions (INDELs) that can lead to functional gene knockout. If a DNA donor template is included, the cell can use those instructions to repair the Cas9-mediated break in a precise manner via the HDR pathway (adapted from 116).

requires two conditions to recognize and cut a specific DNA sequence: 1) a 20-nucleotide (nt) targeting sequence, also known as the protospacer, that makes up part of the CRISPR RNA (crRNA), which along with the trans-acting crRNA (tracrRNA) forms the complete gRNA, and 2) a protospacer adjacent motif (PAM) sequence that lies immediately 3′ of the targeting crRNA/protospacer sequence (Fig. 1). Once these two prerequisites are met, Cas9 will bind the DNA sequence complementary to the crRNA and induce a DSB 3 – 4 nt 5′ of the PAM sequence. Endogenous DNA DSB repair mechanisms will then repair the break. The two most common pathways are: 1) non-homologous end joining (NHEJ), which is the primary repair pathway in most mammalian cells; and 2) homology-directed repair (HDR). While both pathways repair DNA with high fidelity, DNA end processing during NHEJ can result in small insertion or deletion (INDEL) mutations near the cut site. Such INDELs can introduce frameshift mutations and result in truncated and/or non-functional proteins. Under normal circumstances, HDR functions after DNA replication, wherein the second sister chromatid can act as a template for repair. In the context of genome editing, researchers can provide a DNA template with high homology to the endogenous target gene locus that contains the desired genetic change. This incredibly powerful aspect of genome editing can be challenging, as the efficiency of HDR-mediated gene insertion is significantly lower than NHEJ-mediated INDEL formation (*4*), and editing outcomes are a result of the interplay between these two primary repair pathways. Since DNA repair is intrinsically linked to the cell cycle (*2*), cell type-specific cycling kinetics also impact editing outcomes.

THE CRISPR ADVANTAGE

Compared to previous generations of genome editing tools such as ZFNs and TALENs, several outstanding features make the CRISPR-Cas system a robust workhorse in the modern molecular biology lab:

Flexibility

The ever-expanding CRISPR toolbox includes naturally occurring and engineered Cas variants that support a variety of genetic modifications, including precise genome editing, single base editing, transcriptional activation/repression, and epigenome editing. The three main types of CRISPR systems that have been widely repurposed for genome editing (Type II, V, and VI systems) have different PAM sequence requirements; engineered Cas variants with broadened PAM specificity enable editing of a wide range of genetic loci possible.

Accessibility

CRISPR allows researchers to perform precise genome editing due to the relative ease of design and production of the requisite guide RNA sequences to target genomic sequences of interest. The expansive variety of available CRISPR tools enable cost-effective implementation and straightforward design to precisely modify the genetic or epigenetic features of target cells.

Efficiency

CRISPR outperforms traditional genome editing technologies in speed and efficacy, enabling researchers to rapidly generate genetically modified cells or organisms. Examples include 'isogenic' cell lines that have identical genetic information other than the disease-relevant target gene(s), and *ex vivo* editing of human somatic cells for therapeutic applications. CRISPR can also be used to conduct multiplex high-throughput functional screening assays by synthesizing and assembling large collections of guide RNA 'libraries' that can be used to systematically knock out or modulate gene expression.

The features of the CRISPR-Cas system make it an invaluable tool for genome editing and, despite its recent discovery, have already enabled scientists to address basic biological questions and make significant advancements in the development of gene and cellular therapies.

Historical Milestones of CRISPR-Cas Technology

Dr. Francisco Mojica, a microbiologist at the University of Alicante in Spain, coined the term CRISPR when he identified a bacterial genetic locus consisting of repeats interspaced with short sequences that match the bacteriophage genome (***5***). This finding led him to correctly hypothesize that bacteria acquire and store the external phage sequence as part of their adaptive immune system to guard against subsequent challenge. While Mojica's adaptive immunity hypothesis was resoundingly validated by work published in 2007 (***6***), the mechanism by which the CRISPR system inactivates the invading phage and the effector protein(s) involved in the process were not yet clear.

Between 2008 and 2010, the mechanism of CRISPR was found to involve ***Cas*** genes that encode DNA cleavage enzymes within the CRISPR locus (***7***, ***8***). Subsequent studies led by Dr. Virginijus Šikšnys found that purified Cas9 could be reprogrammed to target and cleave genomic sites of choice by changing the guiding CRISPR RNA (crRNA) sequence (***9***, ***10***). The same year, Jennifer Doudna and Emmanuelle Charpentier made a similar discovery that Cas9 is the key factor mediating the DNA cleavage capability of CRISPR (***11***). In the same paper, the duo described the two essential RNA components of the CRISPR system, crRNA and tracrRNA, which could be combined into a single guide RNA (sgRNA) to streamline design and delivery of the CRISPR system. Taken together, these results demonstrated the remarkable feature of CRISPR-Cas9 as a self-contained RNA-guided DNA endonuclease that does not require a cofactor. These discoveries laid the theoretical foundation for application of this novel system across a wide range of cell types and organisms to achieve precise genome editing.

A significant advancement in the application of CRISPR technology was made in 2013 with the release of the PX330 series of CRISPR-Cas9 plasmids developed in Dr. Feng Zhang's lab for gene targeting in eukaryotic cells (12). These tools launched the sweeping popularity of CRISPR tools for a variety of applications, including the first CRISPR-mediated genome editing in human stem cells (***12***) and the first cohort of CRISPR-edited mouse strains (***13, 14***). The use of CRISPR has since been gaining momentum, with exciting discoveries made on a regular basis and new tools constantly being added to the ever-expanding CRISPR toolbox. We will discuss some of the most popular CRISPR tools and their applications in the following sections of this book.

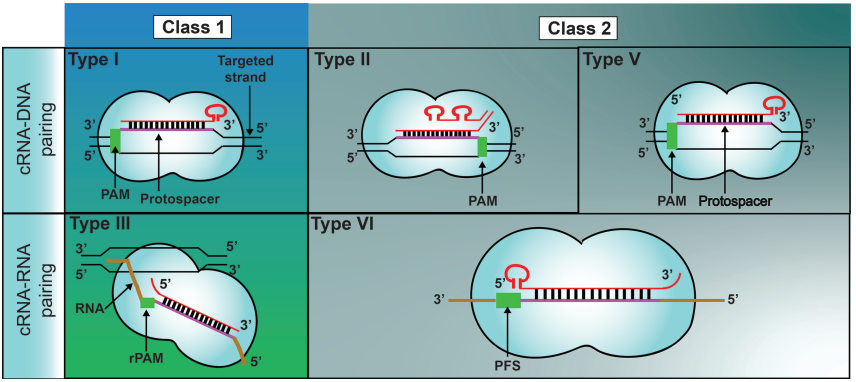
More recently, CRISPR tools have advanced to the point of supporting therapeutic applications. The first experimental CRISPR-based clinical trial was launched in August 2018 by Vertex Pharmaceuticals Inc. and CRISPR Therapeutics to treat the blood disorder beta-thalassemia. In this trial, blood cells will be removed from the patients, edited *ex vivo* to reduce expression of the fetal hemoglobin repressor ***BCL11A***, and transplanted back into the donor patients. The first in-body CRISPR medicine testing was approved by the FDA in November 2018. In this ongoing clinical trial, scientists from Editas Medicine and Allergan are collaborating with medical teams to correct the IVS26 point mutation of the ***CEP290*** gene in the retina that leads to Leber's congenital amaurosis type 10, the most common form of inherited childhood blindness.

Choosing the Right CRISPR System for Your Experiment

The diversity of naturally evolved and protein-engineered CRISPR-Cas variants allows researchers to pick the most suitable tool for their particular genome editing applications (Figure 2). Cas9 is the first and most well-characterized single-protein CRISPR effector. The Cas9 enzyme contains two conserved nuclease domains, RuvC and HNH, which together make a blunt DSB near the PAM sequence (***8***). The DSB induced by Cas9 is then repaired by NHEJ or HDR. NHEJ can lead to INDEL formation and possible generation loss-of-function mutations. Alternatively, when HDR

is engaged by the cell, precise genetic changes can be introduced that are facilitated by co-delivery of a DNA donor template that contains homology arms to instruct the cell to utilize it as a repair template. The type II CRISPR-Cas9 system generally exhibits high on-target genome editing efficiency, but the relatively short gRNA targeting sequence (20 nt) leaves room for off-target cleavages at unintended genomic sites.

In an effort to reduce potential off-target mutagenesis by wild-type Cas9, mutant forms of the protein were generated wherein one of the nucle-



Type	Members	Size (aa)	Guide spacer length (nt)	Total guide length (nt)	PAM	Cut	Primary Application
Type II	Cas9 (Type II-C)	~1000–1600	18–24	~100 (sgRNA)	3-NGG (SpCas9), 3-NNGRRT (SaCas9), 3-NNNNGATT (NmCas9)	Blunt-end DSB	Gene knockout (NHEJ) DNA knock-in (HDR)
Type V	Cpf1 (type V-A) C2c1 (type V-B)	~1100–1300	18–25	42–44	5-TTTN (FnCas12a)	5-nt overhang DSB	Gene targeting of AT-rich locus
Type VI	C2c2 CasRx (type VI-D)	~900—1300	22–30	52–66	3-H (LshCas13a), 5-D and 3-NAN or NNA (BzCas13b), none (RfCas13d)	ssRNA	RNA silencing; transcript detection

Figure 2: Types and classes of CRISPR-Cas9 systems.

Class 1 CRISPR systems require multiple Cas proteins, while Class 2 CRISPR system require a single Cas protein (adapted from 117).

CRISPR-Cas9: clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9; crRNA: CRISPR RNA; PAM: protospacer adjacent motif.

ase domain catalytic residues (D10A in RuvC or H840A in HNH) is inactivated to render the protein capable of cleaving only a single DNA strand (***15***). Single-strand breaks (SSBs) are repaired with higher fidelity than DSBs (***16***), and these Cas9 nickase (Cas9n) enzymes were indeed shown to exhibit lower off-target activity than their wild-type counterparts (***15***). However, the reduced efficiency and more complicated experimental design of this strategy has prevented widespread adoption.

The Type V CRISPR system (Cpf1, Cas12a) employs compact and efficient enzymes that use longer gRNA sequences (typically >23 nt) to create staggered cuts near TTTN PAM sequence with a 5-nt overhang, making them very useful for genome editing in AT-rich genomic regions (***17***). Cas12a cleaves and processes its own guide RNAs through acid-base catalysis, a feature that allows the separation of multiple gRNAs from a single RNA transcript for multiplex genome editing. Once Cas12a binds a target single-strand DNA molecule that matches its spacer sequence, it will activate and indiscriminately cleave all single-stranded DNA near the initial binding site. This property makes Cas12a a powerful tool for detecting tiny amounts of target DNA in a mixture, which was used to develop the method termed DNA Endonuclease Targeted CRISPR ***Trans*** Reporter (DETECTR)(***18***).

The type VI CRISPR system (Cas13) forms a complex with a 30-nt long crRNA which, when bound to a complementary single-stranded RNA (ssRNA) sequence, triggers nonspecific RNase activity near the initial ssRNA binding site (***19***). This unique promiscuous RNase activity has been harnessed ***in vitro*** for precision diagnostics to detect trace amounts of specific RNA transcripts in a technique termed Specific High-RNA-guided RNA sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) (***20***). Editing systems that target RNA transcripts do not induce permanent changes to the genomic DNA sequence and thus are more transient and theoretically safer than their DNA-altering counterparts. Studies suggest that sgRNA-guided gene silencing by Cas13 is significantly more specific than the traditional small-hairpin RNA (shRNA) approach (***19***). Therefore, the Cas13 system could represent a clinically promising gene therapy solution to enable reduced expression of mutant RNA transcripts.

In addition to the type II, V, and VI CRISPR systems that have been harnessed for DNA- and RNA-editing applications, other types of CRISPR systems with different DNA binding and genome-editing properties have also been described, including Type I (***21***) and Type IV CRISPR/Cas systems (***22***). These CRISPR systems are evolutionarily similar but utilize multiple Cas proteins that form complexes with crRNA to guide DNase activity. Type III CRISPR-Cas systems display both RNase and RNA-activated DNase activity (***23***).

Systematic investigation of the naturally evolved CRISPR-Cas systems in different species may lead to the identification of better genome editing tools. For example, the recent characterization of six smaller Cas9 orthologs led to the identification of the ***Staphylococcus aureus*** Cas9 (SaCas9) ortholog that has efficiency and specificity comparable to the commonly used ***Streptococcus pyogenes*** Cas9 (SpCas9) but is substantially smaller, improving the efficiency of cellular delivery (***24***).

Protein-engineered variants of Cas nucleases have also been developed for applications that do not require DNA or RNA cleavage, but instead rely on protein recruitment to specific genomic locations by catalytically dead Cas (dCas9) variants that retain DNA-binding ability (25). These applications include modulation of gene expression, labeling specific genomic loci or RNA transcripts (Fig. 3) (reviewed in ***26***). More recently, Cas enzymes have been adapted for base editing to induce targeted C-to-T or A-to-G transition mutations (***27***-***29***). While early base editors were developed by fusing naturally occurring or engineered deaminase enzymes to catalytically dead Cas9 or Cas12a, the next generation of base editors utilize nickase mutants fused to base repair inhibitors. These examples of clever protein engineering demonstrate the power of basic biochemical studies and highlight the vast utility of CRISPR-Cas systems in many forms of genetic manipulation.

The discovery of CRISPR-Cas systems has spawned an entire field of research dedicated to identification and characterization of naturally occurring CRISPR-Cas systems that can be repurposed for genome editing, as well as ingenious methods to engineer these systems for a wide variety of genetic alterations. While these efforts have advanced the field

at a rapid pace, wild-type Cas9 remains the most widely used reagent for genome editing. In the coming sections we will focus on the CRISPR-Cas9 system although many of the concepts can be extended to other CRISPR-Cas systems.

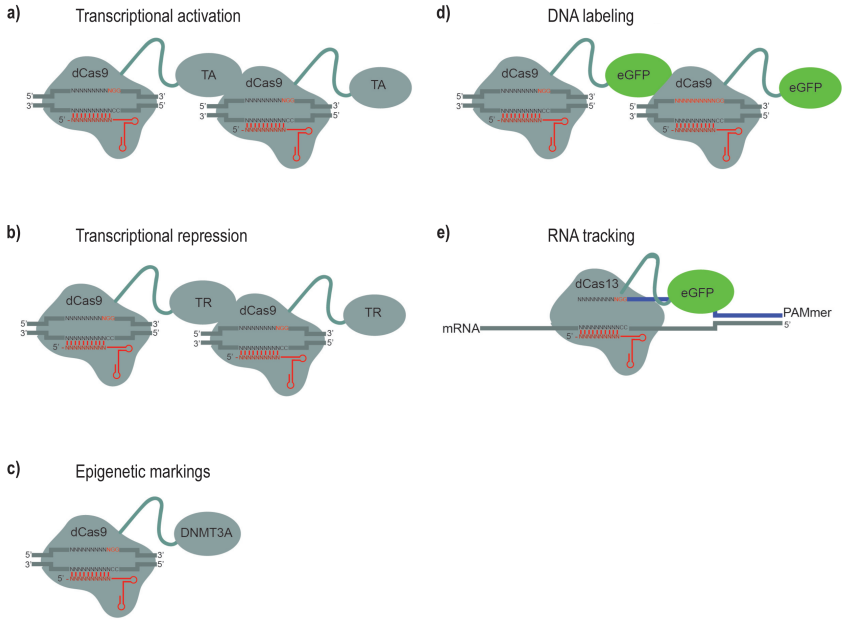


Figure 3. CRISPR-Cas applications beyond genome editing.

(a) CRISPR-Cas9 can be converted into site-specific transcriptional activators by the fusion of dCas9 to transcriptional activators (TA) such as VP16/VP64 or p65 activation domains. Tiling of these site-specific transcriptional devices can modulate gene expression. (b) CRISPR--Cas9 can be converted into site-specific transcriptional repressors by the fusion of dCas9 to transcriptional repressors (TR) such as KRAB or SID, which promote epigenetic silencing. Tiling of these site-specific transcriptional repressors can modulate repression of gene expression. (c) CRISPR-Cas9 can be converted into locus specific epigenetic modifiers through fusion of dCas9 to DNA methylases such as DNMT3A, to DNA demethylases, or to histone acetylases/deacetylases. (d) Fusion of dCas9 to a fluorophore or fluorescent protein, such as eGFP, enables imaging of DNA loci. (e) Transcripts can be imaged by fusing dCas13 to a fluorophore or fluorescent protein, such as eGFP in addition to a small oligonucleotide that provides a PAM sequence (PAM) (adapted from 118).

gRNA Design Strategies

The CRISPR-Cas9 gRNA contains a 20-nt unique crRNA targeting sequence (protospacer) adjacent to a 76-nt backbone sequence that contains the tracrRNA, which acts as a scaffold for the interaction between Cas9 and the gRNA sequence. The Cas9-gRNA complex will scan the genome and bind loci that are complementary to the crRNA/protospacer sequence and harbor a 3′ PAM. Once the complex binds to DNA, Cas9 will induce a DSB precisely 3 – 4 bp upstream of the PAM site, and the choice of DNA repair pathway engagement will dictate editing outcomes.

The gRNA sequences used for gene targeting may be designed with online tools such as CRISPOR (***30***), E-CRISPR (***31***), COSMID (***32***), and others. These tools search for potential gRNA target sequences within the input genomic sequence of interest (typically 30–50 bp surrounding the site of interest) and provide information about the predicted 'on-target' activity and potential 'off-target' activity of each gRNA (see the "Off-target effects of CRISPR-Cas" subsection in the "Current Challenges" section). While these tools are very useful in ranking the specificity and activity potential gRNAs near the region of interest, gRNAs must be tested empirically to determine efficacy as our knowledge of the parameters that define an 'optimal' gRNA sequence is limited (***33, 34***). Since editing efficiency is dependent on cut-to-mutation distance (***35***), the location of the gRNA-directed break should be carefully considered, particularly when the editing outcome is rare (e.g. precise knock-in generation).

CRISPR-Cas Expression and Delivery System Formats

CRISPR machinery can be expressed in target cells using several different methods, including expression plasmids, viral vectors, *in vitro*-transcribed mRNA, or purified protein pre-complexed with the gRNA, also referred to as ribonucleoprotein (RNP) complexes (Table 1). If knock-in is desired, a donor DNA template can be provided in the form of plasmid DNA or single-stranded DNA oligodeoxynucleotides (ssODN or long ssDNA), introduced into the cell concurrently or sequentially with CRISPR-Cas components as naked DNA or packaged in viral vectors e.g.

adeno-associated virus (AAV). The extent of genetic change will inform the type of donor template used: ssODNs are usually ~50–200 nt in size, they can only mediate small genomic changes, while long ssDNA can be generated up to several kilobases (kb) and AAV vectors can be used to insert transgene cassettes up to ~4 kb (***36***).

Depending on the CRISPR-Cas9 expression system chosen, complete gRNAs can be obtained from several sources (Table 1). When using plasmid-based expression, the crRNA/protospacer sequence can be cloned immediately 5′ to the gRNA backbone sequence on a Cas9 expression plasmid, for example, using the PX330 series of plasmids (***12***). These plasmids are designed to express Cas9 and a selectable marker, such as antibiotic resistance or fluorescent proteins to facilitate enrichment of transfected cells. In addition to plasmid-mediated expression of sgRNAs, ***in vitro*** transcribed (IVT) sgRNA can be purified and delivered alongside Cas9 mRNA or purified Cas9 protein. Finally, chemically synthesized gRNA (either separate crRNA and tracrRNA, or sgRNA) can be coupled with purified Cas9 protein to form ribonucleoprotein (RNP) complexes. Chemical modification of synthetic gRNAs can enhance editing efficiency, likely due to reduced endonuclease degradation (***37***).

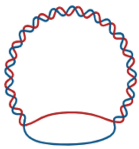
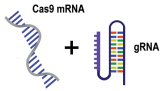
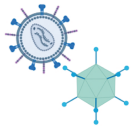
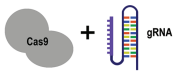
Expression Plasmid 	• Inexpensive • Expandable • Simple delivery to immortalized cell lines	• Genomic integration issues • Delay for transcription/translation of gRNA/Cas9 protein • Prolonged expression resulting in increased off-target effects
In vitro Transcribed (IVT) gRNA + Cas9 mRNA 	• Non-integrating (DNA-free)	• Low stability of RNA • Delay for translation of Cas9 protein • Potential immunogenic effects
Viral Vectors (e.g. Lentivirus, AAV) 	• High delivery efficiency in most cell types • Expandable	• Genomic integration issues • Potentially not applicable for clinical translation, manufacturing challenges • Most Cas variants larger than maximal packaging size
Ribonucleoprotein (RNP) Complex 	• Non-integrating (DNA-free) • Transient and controlled expression • Immediate acting • High editing efficiency in most cell types	• Large protein complexes can be challenging to deliver • More expensive than plasmid-based systems (single use)

Table 1: Comparison of expression and delivery systems for CRISPR-Cas9 and gRNA.

Regardless of how the CRISPR-Cas9 components are expressed, one of the greatest challenges in high efficiency genome editing lies in delivering the components across the cell membrane. A variety of viral and nonviral methods have been derived to achieve successful delivery. Electroporation and chemical transfection are the most commonly used *nonviral* methods to deliver the essential CRISPR machinery into cells in culture. These methods are relatively inexpensive, straightforward, and effective for most stem and primary cell genome editing projects. It is important to consider the cell type of interest prior to choosing an expression and delivery system, as stem cells and many primary cell types are sensitive to manipulation. A pilot assay should be carried out to test the efficacy of candidate CRISPR-Cas delivery and expression methods in the target cell or tissue. In experiments where a limited number of cells are to be edited, for instance oocyte manipulation to develop transgenic animals, mRNA encoding Cas9 or RNP complexes are typically preferred since they can be injected directly into the cell of interest and bypass Cas9 and sgRNA transcription, resulting in faster expression rates and higher genome editing efficacy.

Viral vector-based approaches to deliver components of the CRISPR machinery are typically more efficient than other methods and therefore provide a powerful genome editing tool for both cell culture and *in vivo* applications. The challenge of this approach is that Cas proteins are generally encoded by large genes; high-titer virus preparation is key for the success of this approach, yet suitable viral vectors to encode both the CRISPR effector and gRNA often do not exist. In experimental models such as cell lines or animals, an alternative approach to Cas9 delivery is to engineer an inducible Cas9 transgene into the genome of target cells. Other components necessary for genome editing, such as sgRNAs and DNA repair templates, are much smaller cargo and can be virally packaged and produced at a high titer for efficient delivery in Cas9-expressing cells.

APPLICATIONS OF CRISPR-CAS TECHNOLOGY IN CULTURE SYSTEMS

Genome editing has been successfully applied in numerous cell lines. While many early eukaryotic genome editing studies used immortalized cells, which are easy to culture, manipulate, and clone, more complex cells, such as stem and primary cell types, represent the most scientifically and clinically promising cell types to edit. However, genome editing of stem and primary cells has been hampered by challenges in efficient delivery and expression of the CRISPR machinery, clonogenicity, and cytotoxicity. In the coming sections, we will highlight key milestones that have enabled high efficiency editing in the most difficult-to-manipulate cells and provide example case studies that underscore the versatility and power of CRISPR-Cas genome editing when applied to cell culture systems, particularly for disease modeling and development of cellular therapies.

Next Generation Disease Modeling with CRISPR-Cas

One of the most exciting applications of CRISPR-Cas technology is its use in pluripotent stem cells (PSCs), including embryonic stem (ES) and induced pluripotent stem cells (iPSCs). PSCs have the unique capacity to expand clonally from a single cell, enabling researchers to capture relatively rare genome editing events through single-cell clonal expansion. Induced pluripotent stem cells (iPSCs) are particularly useful as they can be derived from the somatic cells of patients or healthy individuals. However, the high degree of variability between iPSC lines has presented a major challenge in using these cells to study gene function and/or disease-related phenotypes, which is at least in part due to individual genetic variation. Genome editing can circumvent this issue through the generation of isogenic clones that differ only at the genomic site-of-interest but otherwise contain an identical genetic background. Isogenic iPSCs can be differentiated into a wide variety of cell types using two- and/or three-dimensional culture systems, enabling the generation of powerful *in vitro* disease models.

By combining CRISPR and stem cell technologies to introduce or correct putatively pathogenic genetic mutations, side-by-side compari-

son of wild-type and mutant cells can be used to directly interrogate the contribution of certain gene variants to disease-relevant phenotypes. Such approaches eliminate potential confounding factors caused by the genetic background variations inherent in iPSC or ESC lines derived from different individuals and has significantly advanced disease modeling research.

Human PSCs have been especially useful for modeling diseases where the cell type or tissue of origin are inaccessible, such as neurological and cardiovascular disease. Genome-edited human stem cells have been successfully differentiated *in vitro* into a wide variety of cell types of endodermal, mesodermal, and ectodermal lineages such as cardiomyocytes (***38***) and all of the major brain cell types, including neurons (***39***), astrocytes (***40***), oligodendrocytes (***41***), and microglia (***42***). These human cells allow researchers to gain insight into disease mechanisms by studying disease-relevant gene expression, and morphological and functional phenotypes that are directly caused by genetic mutations (thoroughly reviewed in ***43***). Genome-edited human PSC-derived cells can also be used to identify potential therapeutics through high-throughput phenotypic or molecular target-based drug screening platforms.

In addition to two-dimensional cell culture models, another significant application for CRISPR-edited stem cells is the development of three-dimensional (3D) organoid culture systems for disease modeling. Organoids are 3D structures that typically contain a self-renewing stem cell population which differentiates into multiple tissue-specific cell types that exhibit spatial organization similar to that of the organ it represents. Organoids can be derived from adult stem cells (ASCs) or by directed differentiation of PSCs and are capable of recapitulating key functions of their representative organ. These features make organoids a physiologically relevant culture system to study normal and disease physiology and act as diagnostic tools in precision medicine applications.

An exciting class of organoids termed “cerebral organoids” consist of neurons, astrocytes, and cycling progenitor cells that recapitulate characteristic features of human cortical development (***44, 45***). Importantly, knock down of the microcephaly-associated gene CDK5RAP2 in human

brain organoids demonstrated phenotypes representative of the disorder, including several key features that the Cdk5rap2 knockout mouse model failed to recapitulate (***46***). A recent work that combined CRISPR genome editing and organoid technologies allowed researchers to model human-specific aspects of brain development in a dish by demonstrating that knocking out the PTEN gene in human iPSC-derived organoids leads to expansion in organoid size and development of folding structures on the surface (***47***). A similar work utilized brain organoids derived from genome-edited iPSC lines to model glioblastoma tumor formation in a 3D environment (***48***). CRISPR-Cas manipulation of oncogenes/tumor suppressor genes initiates tumorigenesis in cerebral organoids, allowing microscopic observation of tumor development in 3D human brain tissue.

In addition to the brain, the combination of CRISPR, stem cell, and organoid technologies has been applied to disease studies in other organs. Kidney organoids were developed from human PSC-derived epiblast spheroids with GSK3ß inhibition (***49***). The authors showed that CRISPR-mediated knockout of polycystic kidney disease genes PKD1 or PKD2 induces cyst formation from kidney tubules in the kidney organoids, demonstrating that organoids can provide a model of polycystic kidney disease to study the pathogenic process. More recently (***50***), the same group demonstrated that CRISPR-edited PSC-derived kidney organoids can be used for multidimensional phenotypic screening applications. They established a robotic pipeline with liquid-handling machines and high-content imagers to manufacture and analyze kidney organoids in microwell arrays. This type of high-throughput system provides an incredibly powerful platform for drug discovery and development of precision medicine.

Adult stem cells (ASCs) can also be edited using CRISPR-Cas systems and derived into organoids. One example is correction of the mutant CFTR gene in intestinal stem cell organoids derived from cystic fibrosis patients using CRISPR-Cas9 (***51***). Similar ASC-derived organoids were be utilized to model the cell-cell interactions in colon cancer tissue (***52, 53***). Advanced culture systems for primary human airway epithelial cells, which are notoriously difficult to expand long-term, were also recently described (***54***). These methods enable genome editing and subsequent expansion and cul-

turing of the cells at the air-liquid interface, providing an excellent system to study genes involved in airway dysfunction and disease.

The relative ease with which one can genetically manipulate human cells with CRISPR-Cas systems has significantly advanced disease modeling. Coupled with novel culture methods, such as robust differentiation systems and organoid technology, we now have the ability to generate powerful, physiologically relevant human disease models in simple culture dishes to further our knowledge of normal development and disease pathogenesis.

Therapeutic Applications of CRISPR-Cas Technology

In addition to disease modeling, CRISPR-Cas genome editing technology can be applied to perform *ex vivo* editing in either human PSCs or multipotent primary cell types, such as immune cells or hematopoietic stem and progenitor cells (HSPCs), to provide an autologous cell source for clinically relevant cell therapies. Successful cell therapy requires the ability to genetically modify cells with advantageous traits such as the potential for target homing or correction of pathogenic mutations, and subsequent delivery of those cells into a recipient. This requires the availability of suitably matched donors and is hampered by challenges in cell manufacturing and potential immunological complications. CRISPR-Cas enables insertion of single-copy therapeutic genes at precise loci, overcoming the safety limitations of random integration of viral vectors with potential for gene disruption. Moreover, genome editing with CRISPR-Cas enables concurrent editing at the therapeutically relevant locus and other site(s) to prevent immune rejection, a strategy that is rapidly being incorporated into the development of next-generation cellular therapies.

In the field of regenerative medicine, one of the most promising applications of CRISPR technology is the generation of allogeneic or human leukocyte antigen (HLA)-matched human PSCs for cell therapy. Polymorphisms in HLA class I genes lead to high rejection rates of transplanted PSC-derived cells in allogeneic recipients. In a recent report (***55***), an AAV-mediated CRISPR-Cas delivery system was used to knock in a minimally polymorphic HLA-E gene into the Beta-2 Microglobulin (B2M) surface

antigen gene locus. The edited HLA-E-B2M fusion protein-expressing cells could escape immune detection and natural killer (NK) cell-mediated lysis, providing a potential source of universal donor cells for transplant applications.

Primary cells, however, introduce several layers of complexity into a genome editing workflow, as they have limited expansion capacity, low clonogenicity, are difficult to manipulate, and can be sensitive to exogenous DNA and/or unmodified gRNA. Early attempts to apply CRISPR-Cas9 genome editing in primary cell types used either viral vector or plasmid expression systems with limited success, and subsequent studies identified that unmodified foreign nucleic acids can sometimes be detected by the innate immune system, causing cytotoxicity and reduced editing efficiency (***56-58***). More recently, electroporation of cycling primary cells with RNP complexes has achieved high efficacy across a number of targets (***36, 37, 59-62***). However, the limited expansion of primary cell types makes clonal generation extremely challenging. Obtaining high editing efficiency is therefore of utmost importance in these cell types. Various methods have been explored to overcome these difficulties, including culture manipulation to promote cell cycle activation and implementation of novel strategies for delivery and expression of the CRISPR-Cas machinery to evade immune activation.

Chimeric antigen receptor (CAR)-T cell therapy has emerged recently as a very promising cancer therapy approach. CAR-T cells are genetically reprogrammed immune cells designed to specifically target and kill cancer cells. Currently, viral and non-viral methods can be used to introduce the engineered CAR into the T cell genome (***63***). While viral vectors randomly integrate into the genome and therefore pose safety concerns, the CRISPR-Cas system enables CAR integration at precise genomic locations (***64***). Multiplex genome editing of the ***TRAC***, ***B2M***, and ***PD-1*** genes was used to generate knockout T cells that exhibited minimal immunogenicity and inhibitory PD-1 signaling when implanted into a xenograft mouse model (***65***). This strategy was recently advanced through demonstration of non-viral genome targeting with long ssDNA donor templates for CAR integra-

tion (*66*), which can be combined with deletion of inhibitory genes and/or correction of pathogenic mutations to engineer primary human immune cells with enhanced therapeutic properties. The synergy between these two revolutionary technologies, CRISPR-Cas and CAR-T, is accelerating the development of novel immunotherapies and may provide a cell source with low immunogenicity and high efficacy for cancer treatment (*67*).

A recent study showcased the multiplexing potential of the CRISPR-Cas9 system (*68*), wherein a genome-wide CRISPR knockout screen in primary human T cells was used to identify regulators of immune cell function. The method, coined "Single guide RNA (sgRNA) Lentiviral Infection with Cas9 protein Electroporation" (SLICE), identified candidate hits that were shown to boost T-cell activation and *in vitro* cancer cell killing (*68*). This study represents a promising example of how CRISPR-Cas technology can help address basic biological questions that significantly advance development of future cell therapies.

Beyond T cell engineering, another exciting therapeutically relevant area of CRISPR-Cas application is *ex vivo* genome editing of HSPCs for treatment of hematological malignancies and hemoglobinopathies. For example, Mandal et al. (*69*) provided one of the first reports concerning the application of CRISPR-Cas9 in $CD34^+$ HSPCs wherein they used a proximal dual gRNA-targeting approach to delete a clinically relevant gene (*CCR5*). Subsequent studies demonstrated reduced cytotoxicity and improved editing efficiencies could be achieved in HSPCs by using an RNP-based expression system (*37*, *70*).

While genetic knockout in HSPCs is relatively straightforward, the repopulating population of HSPCs, long-term hematopoietic stem cells (LT-HSCs), exhibit low HDR frequencies and a slow cell cycle (*36*, *71*). This presents a major hurdle in HSPC knock-in editing for therapeutic purposes. To overcome this limitation, the Porteus group has been working to identify improved genome editing methods and optimized culture conditions to prime HSPCs for high efficiency HDR-dependent knock-in editing (*72-74*). These methods include culture in cytokine-rich media to support expansion of cycling progenitors and the use of AAV6 to package and deliver the

DNA donor template. In one study, an AAV6-packaged repair template was used to correct a pathogenic sickle-cell disease Glu6Val mutation in the ß-globin (*HBB*) gene (**72**). Through insertion of a GFP reporter cassette, the authors were able to enrich successful HDR-edited cells. Importantly, the *HBB* gene-targeted HSPCs display long-term and multilineage reconstitution in immunodeficient mice. Other donor DNA templates beyond AAV6, including long ssDNA or short ssODNs, may also prove useful in the context of HSPC editing. While ssODNs mediate efficient HDR in HSPCs (**73**), they are not long enough to encode a selection cassette and therefore are not compatible with the enrichment protocol described above. However, a recent study identified that genome editing is accompanied by a transient p53-dependent DNA damage response, which is exacerbated by co-delivery of AAV6 but not ssODN (**71**). This exacerbated and sustained p53-dependent DNA damage response correlated with reduced proliferative capacity and functional impairment that could be overcome with transient p53 inhibition.

Significant progress has been made in applying CRISPR-Cas editing systems to clinically relevant cell populations. With continual development of safer and more efficient methods for precise genome editing in primary and stem cell types, there is no doubt that we will witness its successful transition into the clinic in the years to come.

Current Challenges and Troubleshooting

Despite advancements in applying genome editing to the generation of advanced cellular models and therapies, there remain significant hurdles to overcome before the potential of these tools and techniques can be fully realized. These next sections will outline some tips to troubleshoot CRISPR experiments, discuss outstanding challenges, and describe methods being employed to overcome them.

Obtaining High-Efficiency Genome Editing with Confidence

The primary challenge of genome editing is to generate on-target DNA changes at high efficiency, e.g. obtaining a high percentage of cells carrying the intended mutation in the total edited cell population. A number of factors impact genome editing efficiency, including gRNA efficiency, cell type-dependent differences between NHEJ and HDR repair efficiency (concepts discussed in the 'A Brief History of Precise Genome Editing Technologies' section), cell culture conditions that affect cell viability and functionality, and different systems of CRISPR component expression and delivery that may work at different efficiencies in different cell types.

To troubleshoot a CRISPR experiment, one should begin by confirming the design and sequence of the gene-targeting plasmids or synthesized RNA. For NHEJ-based gene knockout experiments, if a single gRNA does not induce editing at the target locus efficiently enough, consider using multiple gRNAs to target multiple genomic loci within 100–200 bp proximity to increase the probability of gene disruption. For HDR-dependent precise genome editing, the designed sgRNAs should be able to cut the unedited genomic DNA sequence but not the repair template or the successfully edited gene locus. If necessary, silent mutations should be introduced to disrupt the PAM or sgRNA sequence on the repair template. In addition, the efficiency of DNA template incorporation in HDR is dependent on cut-to-mutation distance (***35***). The location of the gRNA-directed DNA break should be carefully considered for the generation of precise knock-in edits.

The transfection efficiency of CRISPR components, especially the expression of Cas proteins in the cell type of interest, determines the success rate of genome editing experiments. Candidate methods to deliver CRISPR-Cas components should be tested in parallel in the cell type/tissue of interest to determine the optimal method for subsequent experiments (refer to the 'CRISPR-Cas9 Expression and Delivery System Formats' section). If antibiotic resistance or FACS-based selection is used to enrich the transfected cell population, test the target cells' response to different doses

of antibiotics or optimize the gating strategy of the FACS experiments to validate the sensitivity and effectiveness of the enrichment strategy.

In order to faithfully detect genome editing events, the specificity and sensitivity of the genotyping strategy should be tested and optimized before the actual gene targeting experiments. A general PCR genotyping strategy is to design primers to amplify relatively small (200–500 bp) DNA fragments that flank the target site and/or span the junctions between the inserted gene and the edited genomic sequence (Fig. 4 A). Avoid designing large (>1 kb) amplicons with both primers on genomic DNA as the PCR efficiency for large bands from genomic DNA is relatively low and may result in false negative results. In the case of introducing mutation(s) through genome editing, one potential genotyping strategy is to design the donor template to insert intended mutations and potentially other silent mutation sites in a way that restriction enzyme sites are introduced or disrupted near or at the intended point mutation. With this design, the target region PCR amplicons obtained from edited and unedited cells can be genotyped by restriction digestion. In order to confirm incorporation of large knock-in sequences at the target locus, long-range PCR and/or Southern blot assays can be designed to detect the number of transgene integration events in the targeted cell population (Fig. 4B).

Ensuring High Quality Edited Cells for Downstream Experiments

An important consideration when working with and manipulating stem and progenitor cell types is the maintenance of cell identity. Long-term culture can result in spontaneous differentiation and loss of potency. Moreover, the genome editing workflow is stressful and imparts a high degree of selective pressure, most notably during post-editing survival. As genome editing typically relies on induction of a DNA DSB, in most cell types the p53-dependent DNA damage response will become activated during the process to induce cell cycle arrest or apoptosis. This has been observed in both human PSCs (***75-77***) and HSPCs (***71***). The cellular response to genome editing remains poorly characterized but may negatively impact key features of stem and primary cells such as survival, proliferative capacity, potency, and downstream function. It is therefore important

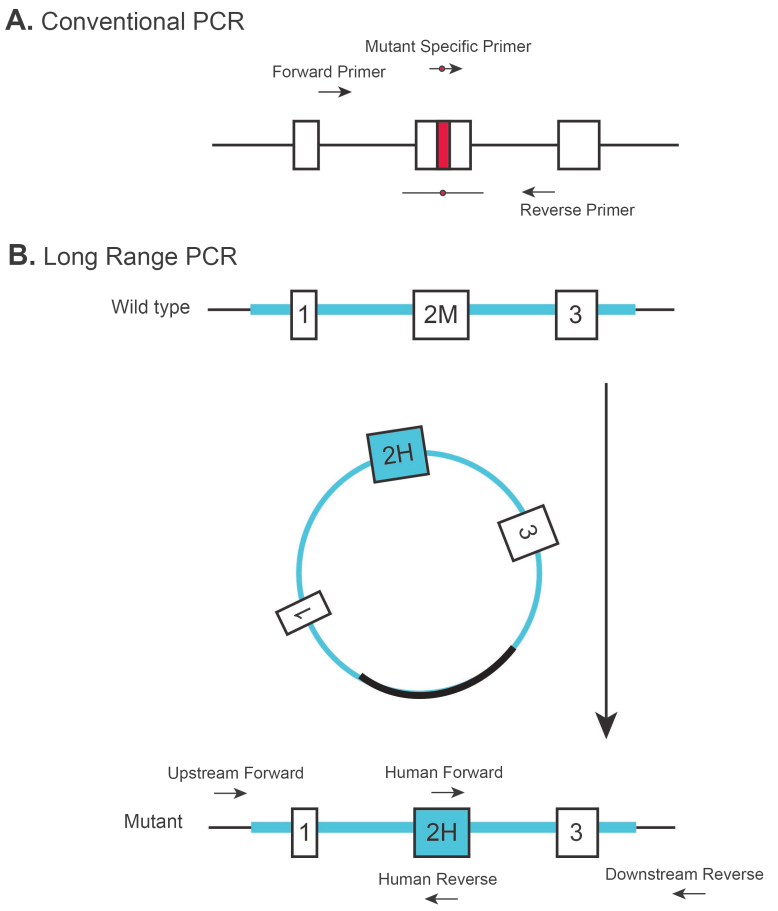


Figure 4: Genotyping strategies to verify genome editing success.

(A) Genotyping by conventional PCR. Primer pairs flanking the gRNA binding site are used to amplify the target region. Assays such as the T7 Endonuclease I or Surveyor assay can be used to estimate the proportion of INDELs generated i.e. cleavage efficiency. For knock-in, mutant-specific primers can be designed to amplify only the mutant allele.

(B) Genotyping by long-range PCR. If a large knock-in is desired, mutants can be genotyped with primers targeting sites outside the homology arms and within the transgene/knock-in sequence (adapted from 119).

to monitor these aspects of cellular health after genome editing and prior to downstream experimental use. Optimization of pre- and post-editing culture conditions can help minimize experimental variability to support generation of functional and viable edited cells.

Beyond p53, additional apoptotic pathway factors can act to limit human PSC survival post-editing. For example, overexpression of the anti-apoptotic protein BCL-XL greatly enhanced editing efficiency (***78***). This finding is quite pertinent, as long-term culture of human PSCs is associated with acquisition of genetic abnormalities, including dominant negative p53 mutations (***79***) and amplification of 20q11.21, the genomic region containing the ***BCL-XL*** gene (***80, 81***). To ensure cells do not acquire unwanted genetic mutations, continual monitoring of human PSC genomic stability is thus essential during both during long-term maintenance and after the genome editing process.

Another challenge for CRISPR experiments in cultured cells lies in the generation of clonal cell lines due to low survival rates after genome editing procedures and loss of proliferative capacity. As the efficiency of a given editing event is never 100%, genetically mixed cell populations are generated after editing. In these cases, one can dissociate single cells and replate at very low density to clone a genetically homogeneous population of cells. However, this approach is only applicable to cell types that are amenable to cloning. Optimization of pre- and post-editing culture conditions can help minimize experimental variability to support generation of functional and viable edited cells.

Off-target Effects of CRISPR-Cas Technology

Genome editing may induce off-target effects that are both sequence-dependent and -independent, including activation of the immune and/or DNA damage response (covered above), as well as mutation(s) at unintended locations in the genome. Cas9 can tolerate ~1–2 bp mismatches in gRNA-DNA sequence (***82***). Along the length of gRNA, PAM-proximal mismatches have been observed to be less well tolerated than PAM-distal mismatches (***83, 84***). Most gRNA design tools account for this when rank-

ing potential gRNAs for a given target; however, it is important to assess potential sequence-dependent off-target effects using unbiased or targeted genomic assays to ensure genome editing specificity. Sequence-independent off-target effects are more challenging to identify but have the potential to negatively impact cell viability and function.

In the cell clones that pass the initial genotype screening, PCR amplification of the target genomic site followed by DNA sequencing can be used to verify the accuracy of the on-target genome editing including mutation and/or transgene insertion. To rule out off-target mutation events caused by CRISPR-Cas in gene-edited cells, PCR amplification and sequencing of potential off-target sites predicted by gRNA design programs is the most accessible method to identify the presence of off-target edits. More systematic and unbiased methods utilizing genome-wide sequencing to detect potential off-target mutations are available, including genome-wide unbiased identification of double-stranded breaks enabled by sequencing (GUIDE-seq) (***85***), high-throughput, genome-wide, translocation sequencing (HTGTS) (***86***), direct ***in situ*** double-stranded DNA (dsDNA) Breaks Labeling, Enrichment on Streptavidin, and next-generation Sequencing (BLESS) (***87***). Whenever possible, it is advised to generate multiple independent clones from one or multiple rounds of gene targeting for further characterization. In addition to genotyping at the DNA level, researchers are encouraged to validate that the observed phenotypes from gene-edited cells match what is expected from the genotype.

WHAT'S NEXT FOR CRISPR?

Expanding the Toolbox

Off-target effects of CRISPR-Cas genome editing has been a major concern and direction for improvement for both the basic and translational research field. Nonspecific DNA cleavage is a consequence of the RNA guide interacting with DNA sequence(s) with imperfect similarity to the target site. This interaction with non-target DNA is stabilized by residues located between the HNH-, RuvC-, and PAM-interacting domains of Cas9 (***88***).

To improve the efficiency, specificity, and targeting range of CRISPR tools for genome editing applications, protein engineering guided by structural information, bacterial selection-based directed evolution, and combinatorial design has led to development of several Cas proteins with enhanced properties (***89-92***). This includes a high-fidelity Cas9 protein (SpCas9-HF1) with nearly undetectable off-target editing (***89***), and an "enhanced specificity" SpCas9 (eSpCas9) variant with reduced off-target editing while maintaining on-target cleavage (***90***). The Doudna group identified enhanced proofreading as the mechanism underlying improved gene targeting accuracy of those novel Cas9 variants, and structural insights led to the development of a hyper-accurate Cas9 variant (HypaCas9) that shows high genome-wide specificity without compromising on-target activity (***91***). Protein engineering efforts have also been focused on broadening PAM specificity, as the ability to edit the genome using Cas9 is limited to regions containing 5′-NGG-3′. By subjecting the PAM-interacting domain of Cas9 to random mutagenesis, Kleinstiver et al. (***93***), identified mutants whose PAM specificity changed from the canonical PAM sequence to 5′-NGA-3′, 5′-NGAG-3′, and 5′-NGCG-3′, thereby broadening the targeting range of Cas9. A modified ***Francisella novicida*** Cas9 (FnCas9) that has less restrictive PAM from 5′-NGG-3′ to 5′-YG-3′ was developed through a structure-guided protein engineering approach similar to those mentioned above (***94***). More recently, the Liu lab engineered a Cas9 variant termed "xCas9" that shows high specificity and the broadest PAM compatibility reported to date (***95***). Similar approaches were recently extended to Cas12a and Cas9 proteins

from additional species (***96***), suggesting these methods can be used to engineer a wide range of Cas enzymes with enhanced properties.

Parallel to work improving the fidelity and to broaden the scope of genome editing, significant efforts have been made to optimize methods for HDR-dependent knock-in editing (***97***). To overcome the low efficiency of HDR, some groups have used small molecule inhibitors and/or activators to promote HDR and suppress NHEJ (***98-103***), or to synchronize the cell cycle (***104***). More recently, physical coupling of the donor DNA template and CRISPR-Cas machinery demonstrated increased HDR frequencies (105-108) that bypass the possible toxicity associated with pharmacological manipulation. Alternatively, others have opted to enrich for HDR-edited cells through positive/negative selection of marker genes (***36, 109, 110***). An example of this type of strategy was recently employed in HSPCs using RNP-based delivery of CRISPR-Cas9 coupled with packing of the donor DNA template in AAV6 to accommodate a GFP reporter gene (***36***). This enabled enrichment of precisely edited cells by fluorescence-activated cell sorting (FACS). A similar strategy was employed in human PSCs to enable precise genome editing (***110***).

Newly developed CRISPR tools that regulate gene expression without cutting the genomic DNA, such as repurposed Cas13 proteins that influence target RNA stability (***19***) and some classes of base editing enzymes, have also attracted attention as novel avenues for genome editing and gene therapy applications.

Improved Delivery and Expression Systems

Despite significant advances in tools for genome editing, one of the greatest challenges lies in successful delivery and expression of the editing machinery in the target cells or tissue. ***In vivo*** delivery is particularly challenging, wherein careful regulation of Cas9 duration and dosage in the correct cells is essential to mediate both the desired therapeutic effects and minimize negative side effects (***111***).

Delivery systems are not broadly applicable across genome editing applications. For instance, electroporation is a relatively effective delivery method for generation of *in vitro* cellular models, yet it cannot be used to deliver the components *in vivo*, and while AAV vectors have achieved success for both *in vitro* and *in vivo* delivery, their packaging limitations and manufacturing challenges preclude widespread clinical usage. A delivery vehicle that can target the desired cells with high specificity and efficacy is crucial. Recent attempts to conjugate RNPs to surface receptors for cell type-specific uptake are promising and demonstrate the possibility of transfection-independent delivery (***112***). Although this method may eventually allow for targeted *in vivo* delivery of genome editing components, it is currently limited by endosomal/lysosomal degradation of the complexes immediately after uptake (***112***).

The use of RNP complexes, in which purified Cas9 protein is precomplexed with in vitro-transcribed or synthetic gRNAs, has gained popularity in recent years. RNP complexes are immediate acting, exhibit transient expression, and are DNA-free. These features reduce the risk of off-target effects, including Cas9-mediated damage at nontarget sites and random integration of plasmid DNA (***113, 114***). Engineering of inducible Cas9 systems represent an alternative strategy to limit the duration of Cas9 exposure to the target genome. An example of this approach split Cas9 into two inactive fragments that could reconstitute into a catalytically active Cas9 protein with the addition of rapamycin (***115***). Development of CRISPR-Cas systems for genome editing purposes have progressed at a rapid pace since their discovery. An enormous amount of effort is currently dedicated to broadening the scope of both research and therapeutic applications through improved genome editing efficiency and specificity. In the coming years, new approaches to precise genome editing, optimized delivery methods, and enhanced genome editing tools will be instrumental in progressing this exciting technology from bench to bedside and beyond.

Looking Ahead

Since its debut in 2013, CRISPR genome editing technologies have advanced basic biology and biomedical research in several ways. CRISPR technologies have made it possible to edit disease-relevant genes in cells and organisms to generate disease models, to edit genes *ex vivo* in primary cells such as HSPCs or T cells as a source for cell therapies, and holds great potential for *in vivo* somatic gene therapy applications. These applications are only a fraction of what is already possible for this emerging technology, yet several challenges must be addressed before it can realize its full potential. Despite these challenges, a global effort to harness the power of naturally evolved CRISPR-Cas systems to alter the code of life in a programmable manner has just begun and is predicted to deliver more amazing results in the years to come.

With all the advancements and successes in CRISPR technology, extreme caution and tight regulation should be applied to the use of such technology when it comes to genome editing in human germline (reproductive) cells. This application is vastly different from genome editing of *in vitro* cultured human stem cells, *ex vivo* manipulation of human cells for therapeutic purposes, and even *in vivo* gene therapies targeting human somatic cells. Genome editing that targets somatic cells is more exact in terms of spatial and temporal precision, which is the basis of modern gene therapy. On the other hand, targeting reproductive cells is not only of little clinical justification (screening of IVF embryos could provide healthy babies without the risk of altering genes), but also will inevitably result in the propagation of both on- and off-target edits in all cells of the individual for its entire lifespan. Further, the modifications will be passed on to any potential offspring and enter the genetic pool of the human population. The potential impact of such an event is currently unable to be determined, and should be addressed through scientific, ethical, and legislative discourse.

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