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CRISPR/Cas9 Technology Improvements and the RNA-Editing Trend

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Abstract

LentiCRISPR/Cas9-V2 is one of the most popular gene-editing and knockout (KO) tools. The lentiviral vector V2mO (CRISPR/Cas9-V2-mOrange) added a visual marker for the easy monitoring of lentiviral production, transduction efficiency, and cell sorting. It provides the estimation of gene editing efficiency by simple calculation of aberrant cells from total cells based on a PCR electropherogram from a cell pool, and it also details a method of gene rescue by overcoming Cas9 editing to KO essential genes, doxycycline-inducible (Dox) lentiviral systems may be used to maintain transduced cells viable while Dox-induced treatment is used to study downstream effects in genes of interest. Low levels of Dox induction may abate Cas9 overexpression in order to reduce off-target editing. A new trend of employing RNA editing without genome alteration using Cas13, ADAR and APOBEC1 has become a hot topic, as these editing techniques may hold the promise of altering RNAs by a single nucleotide conversion or even introducing stop codons, although those systems need further improvements and discoveries.

Keywords CRISPR/Cas9; V2mO improvement; Gene knockout; Dox-inducible Cas9; Essential genes; On-target and off-target editing; RNA editing; Cas13; ADAR; APOBEC1

Abbreviations

ADAR: adenosine deaminase acting on RNA; APOBEC1: apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; Dox: doxycycline; eGFP: enhanced green fluorescent protein; FACS: fluorescence-activated cell sorting; GFP: green fluorescent protein; gRNA: guide RNA; HDR: homology-directed repair; IRES: internal ribosome entry site; KO: knockout; NHEJ: non-homologous end joining; PAM: protospacer adjacent motif; REPAIR: RNA editing for programmable A-to-I replacement; RESCUE: RNA editing for specific C-to-U exchange; RFP: red fluorescent marker; rtTA: reverse tetracycline-controlled transactivator; TRE: Tet-responsive element.

Introduction

CRISPR/Cas9 is a powerful, easy-to-design, cost-effective, versatile, and efficient gene-editing and knockout (KO) tool. In both 2013 and 2015, Science selected it as the Breakthrough of the Year, and in 2017 the journal heralded the precise base-editing technique of a variant CRISPR/Cas system by giving it the same honor [1]. Even though three versions of pLentiCrisprV (V1, V2, and V3) have been developed in the field of gene editing and disruption, the second version pLentiCrisprV2 still remains the most popular and widely used vector for gene function studies (Figure 1). However, many gene KO experiments with LentiCrispr/Cas9, regardless which version used, have failed in research labs. The reasons for the failures may include less optimal condition of HEK293T cells for viral packaging, low titers of lentiviral Cas9 for cell transduction, less optimal gRNAs, a lack of good selective markers for the selection of Cas9-expressed cells, low transduction efficiency for primary cells or primary-like cells, and unedited or wildtype (WT) or WT-like cells overtaking over edited cells [2].

DNA repair mechanisms and challenges of CRISPR/Cas9 systems

There are two major cellular mechanisms to repair genomic DNA damage after the genome is subjected to nicks or double-stand breaks (DSB) **(Figure 1)**.

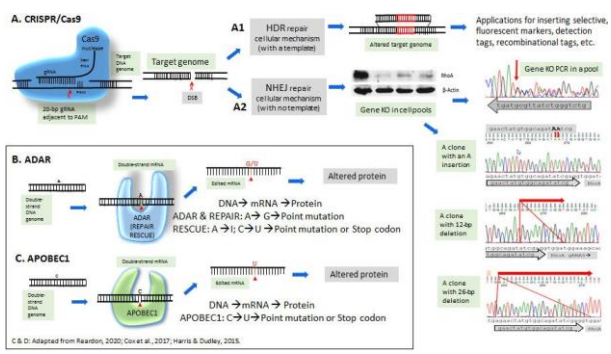

Figure 1: Schematics of DNA- and RNA-editing systems; A. The DNA-editing CRISPR/Cas9 system. DNA cleavage caused by CRISPR/Cas9 is followed by A1: HDR repair, usually from a complementary DNA strand or an introduced DNA fragment; A2. NHEJ repair, which is attributed to gene KO via indels or fragment deletions. B. The RNA-editing ADAR system, in which the nuclease Cas13/ADAR2 fused enzyme is capable of converting adenosine to guanosine (A→G) and cytidine to uridine (C→U) in mRNA; C. The RNA-editing APOBEC1 system, which is capable of converting cytidine to uridine (C→U) in mRNA.

The first is homology-directed repair (HDR), in which cellular machinery uses a homologous template, usually from a complementary DNA strand or an introduced DNA fragment, to fix a nick or DSB (Figure 1A). Researchers have used HDR-mediated repair to insert a DNA fragment with a promoter and GFP marker flanked by two homologous sequences into a site-specific locus [3]. Other researchers have used a single-stranded adeno-associated virus (AAV) for delivery of the donor DNA into a specific locus [4]. Using HDR for gene integration in conjunction with CRISPR/Cas9 and a donor DNA is particularly useful in some types of cells, such as embryonic stem (ES) cells, in which *in vitro* designed gRNAs, exogenous Cas9 protein, and a donor DNA fragment with the intended markers flanked with two homologous arms are directly injected into ES cells to facilitate genome editing [5]. Currently, using commercially available *in vitro* synthesized gRNAs and mass-produced Cas9 protein for HDR genome editing is affordable. Delivery of long DNA fragments via HDR remains the biggest challenge, however, this can be overcome in many ways, such as by using adeno-associated virus (AAV)-based delivery of donor DNA templates [6]. For cell lines, using lentiviral expression of gRNA and Cas9 protein to disrupt a gene of interest, and in meantime to insert a modified DNA fragment into a locus adds significant difficulty because the delivery of lentivirus-expressed gRNA and Cas9 protein and the exogenous introduction of a donor DNA fragment must occur at the right time and in the right place (the nucleus), since lentiCRISPR/Cas9 could deliver the former two, delivery of the latter must concurrently happen in separate DNA vehicle into the nuclei.

A second mechanism for repairing DNA damage is non-homologous end joining (NHEJ) repair, which is attributed to CRISPR/Cas9 gene KO when a homologous template is absent (Figure1A). During the error-prone NHEJ repair process, any mismatch or frameshift of the coding DNA sequences (i.e., 1- or 2-nt indels, fragment deletions at cut sites) would disrupt the expression of the gene of interest. NHEJ repairs resulting in in-frame changes would still be detectable by Western blotting (WB) if the antibodies are designed for the C-terminals of proteins. Our findings revealed that in cell populations with few gene-edited cells, wildtype (WT) or WT-like cells would outgrow in the cell pools, leading to gene KO failure [2]. Thus, stable KO clones established by selective markers and single cloning or single-cell cloning of cells from transduced pools are important to ensure KO success [2].

Some challenges are associated with the current CRISPR/Cas9 system. For example, Cas9, when overexpressed, tends to cut many unintended (off-target) sites on the genome. The literature demonstrates that off-target activity of Cas9-gRNA is present in more than 50% of mice [7]; an overexpressed Cas9 cuts sites with up to five-basepair mismatches to gRNA's 20-basepair binding [8]; and that, in one study, Cas9 cleaved 15 off-target sites from 27 different single gRNAs [9]. Researchers have developed many systems to reduce off-target editing, enhance on-target specificity. One such system, the doxycycline (Dox)-inducible system, offers several advantages over the other systems. Specifically, it is easy to use and dosage-dependent Dox-induction correlates with the Cas9 expression level, thus reducing off-target edits. Moreover, work on prokaryotes is now underway to identify novel CRISPR/Cas editing enzymes that can lead to the development of gene-editing tools that offer more precision and fidelity than those currently available.

In addition, researchers have found that LentiCrispr/Cas9-V1, -V2, and -V3 (two-vector systems) do not work for essential genes, as disrupting the functions of these genes usually leads to cell death. Thus, studies of essential genes require the use of inducible or conditional CRISPR/Cas9 systems. The Tet-On Dox-inducible system is useful in that regard because its second- and third-generation technology provides tighter induction than earlier versions and decreases the leaking of Cas9 basal expression. Another innovative approach to the study of essential genes is the use of RNA-editing systems, which are currently under development. RNA-editing enzymes act on mRNAs or invading viral RNAs via single-nucleotide conversions including stop-codon insertion without genomic alteration. Because RNA editing is reversible, RNA-editing systems bring great promises in essential gene editing. However, they are still in the early stages of development and some hurdles must be overcome, such as Cas13's off-target editing of unintended transcripts [10].

Improvements in the CRISPR/Cas9 system

Construction of an improved lentiviral V2mO vector

We have reported on use of the new lentiviral V2mO vector that offers improvements over the popular pLentiCrisprV2 vector [2]. V2mO, which is derived via insertion of the fluorescent marker mOrange into pLentiCrispr-V2, makes viral production and target-cell transduction visible. Specifically, HEK293T and target cells fluoresce, indicating functional Cas9

expression. Figure 2 shows a schematic flowchart for CRISPR/Cas9 gene KO followed by gene rescue using V2mO. The detailed sequential steps are illustrated in **(Figure 2)**.

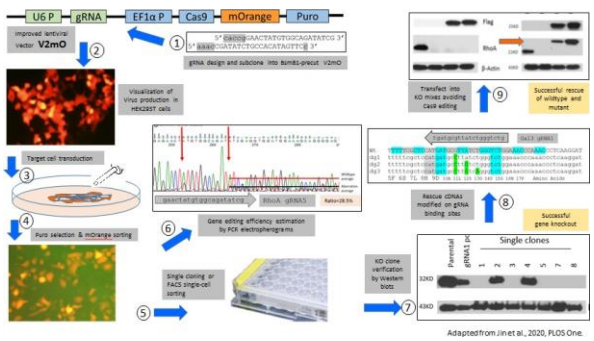

Figure 2: Schematic flowchart of CRISPR/Cas9 gene KO and gene rescue using V2mO. The sequential steps in gene KO consists of; 1. gRNA insertion into V2mO precut with BsmB1; 2. viral production in HEK293T cells; 3. target-cell transduction; 4. target-cell puromycin selection and mOrange sorting; 5. single cloning or single-cell sorting; 6. direct estimation of gene-editing efficiency via analysis of Sanger electropherograms of PCR products; 7. gene KO verification via Western blotting; 8. gene-coding DNA modified by three nucleotides where gRNAs bind to DNA; and 9. rescue or re-overexpression of the genes of interest in KO mixes.

Worth mentioning is that gRNA is ligated and inserted into BsmB1-precut V2mO. Direct estimation of gene-editing efficiency in target cell pools are Sanger electropherograms of short PCR products surrounding gRNA binding sites. Rescue or reoverexpression of the genes of interest to overcome constitutive Cas9 editing requires modifying coding DNA by three nucleotides where gRNAs bind [2].

Other improved lentiviral CRISPR/Cas9 vectors

Many other lentiviral CRISPR/Cas9 vectors are in the public depository Addgene (Cambridge, MA), such as pSpCas9(BB)-2A-GFP (Addgene #48138) [11], pCas9_GFP (#44719) [12] and pCas9D10A_GFP (#44720) [12], pL-CRISPR.EFS.GFP (Addgene #57818) [13], and LentiCrispr-RFP657 (Addgene #75162) [14]. Of these vectors, those with GFP and RFP provide good indicators of both viral titer and clone selection for target cells expressing Cas9 by FACS. However, puromycin selection is one of the most affordable and rapid methods of Cas9-positive clone selection, replacement of the puromycin gene with fluorescence may also be inconvenient for some laboratories because they may have no access to a flow cytometer for cell sorting. In contrast, our V2mO vector contains both fluorescent and puromycin-selection markers, it enables researchers to perform not only inexpensive and fast puromycin selection but also visual single-clone isolation.

To the best of our knowledge, the most important factors in the successful production of high-titer lentiviruses are an exponential HEK293T cell-growth stage (indicating transformation competence) and the presence of visible markers, such as GFP and RFP in vectors.

Dox-inducible CRISPR/Cas9 systems for essential gene KO

Essential genes encode critical proteins needed for central metabolism, DNA replication, mRNA translation into proteins, cellular structure maintenance, and cell transport processes important to cell survival. In contrast, nonessential genes encode proteins that convey selective advantages and increased fitness for organisms and cells to survive and adapt. Simply, direct KO of an essential gene will cause cells to die. Our research projects working with essential genes, such as Trim28 and GRK3, motivated us to adopt the Dox-inducible system for essential gene KO.

Increasingly, researchers have used inducible CRISPR/Cas9 systems in several different fields to identify essential genes. For example, genome-wide screening with an inducible CRISPR system identified ZIC2 as an essential gene for human heart progenitor cells [15]. Yilmaz et al. (2018) [16] employed inducible CRISPR/Cas9 to make a loss-of-function library targeting 18,166 protein-coding genes in haploid human pluripotent stem cells (hPSCs), and found that hPSC-enriched essential genes mainly encode transcription factors and proteins and that a quarter of the nuclear factors are essential for normal growth. In addition, Barger et al. (2019) [17] developed a Dox-inducible system, TLCV2 (Addgene #87360), for essential gene KO. In this system, U6-promoter-gRNA, a selective marker puromycin, inducible Cas9, and eGFP were all integrated into a single lentiviral vector. This vector used a second-generation Tet-On system **(Figure 3A)**.

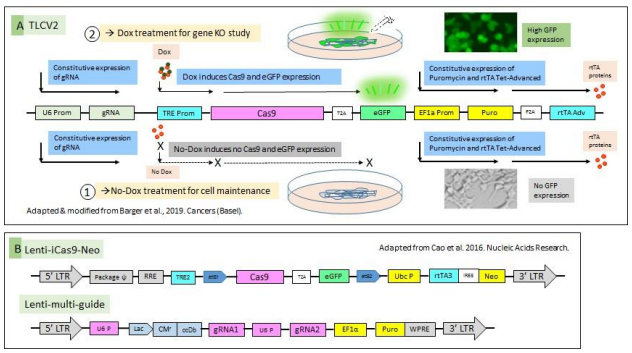

Figure 3: Lentiviral Dox-inducible systems.
A) All-in-one TLCV2 for studying essential genes: 1) Treatment 1, the no-Dox treatment for cell maintenance, suppresses Cas9 and eGFP and keeps the gene of interest unedited; 2) Treatment 2 is the gene KO experiments where Dox treatment induces expression of Cas9 and eGFP, which cuts the gene of interest with a gRNA.
B) The two-vector system (Lenti-iCas9-Neo and Lenti-multi-guide) was designed to abate Cas9's off-target effects and KO multiple genes with similar cellular functions. This third-generation Tet-On system uses two separate lentiviral vectors: one uses inducible Cas9 and eGFP with the selection marker neomycin, and the other uses multiple gRNAs integrated via ligation of BsmB1's unique flanking sequences, which carries the puromycin **selection marker.**

In this system, gRNA, puromycin, and rtTA protein (a Tet-On component) are constitutively expressed, but the TRE remains turned off. When Dox is added to the medium, Dox combines with rtTA protein to turn on Cas9 and eGFP expression. As illustrated in Figure 3A, cell maintenance treatment involves suppressing Cas9 and eGFP expression in order to prevent the editing of essential genes and to maintain cell survival without Dox treatment. Gene KO involves adding Dox to induce Cas9 and eGFP expression, Cas9 then edits the gene of interest with the gRNA binding to specific locus.

In the TLCV2 system, when essential genes are being studied, transduced cells are kept alive without induction of Cas9. When Dox is added to medium for a preset period of time, its downstream effects on essential genes can be studied before cells start to die of essential gene disruption. A second advantage of the inducible system is that if the efficiency of both lentiviral transduction and target gene KO is high enough, a cell pool or population would be acceptable for studying downstream effects without the need for single cloning.

A drawback of the TLCV2 system is that the vector does not have an upfront visible marker that can be used to quickly estimate lentiviral production in HEK293T cells and to determine later lentiviral transduction in target cells. Empirically, we have noticed that visible marker expression is one of the most important markers for viral production and gene KO experiments. Leakage (Cas9 basal expression) is another challenge associated with the inducible system. Expression of Cas9 and eGFP will be expressed at low level depending on how tightly an inducible system is constructed with regard to rtTA and TRE. Second, how much residual tetracycline level in fetal bovine serum (FBS) of growth medium poses significant impact.

Dox-inducible Cas9 systems for abating off-target effects of CRISPR/Ca9

An early inducible Cas9 system developed at Yale University controls Cas9 activation and eGFP expression [18] via a third-generation Tet-On system in which rtTA3 is used and a Cas9-P2A-eGFP cassette is controlled by TRE2 **(Figure 3B)**.

The study demonstrated a significant reduction in expression of several genes of interest across several cell lines and that the inducible CRISPR/Cas9 system dramatically decreased off-target effects of CRISPR/Ca9. This is a powerful, multi-gene inducible KO system involving two-component Lenti-iCas9-Neo and Lenti-multi-guide, particularly when used for several genes with similar functions. However, several challenges are associated with this system. First, eGFP, like Cas9, is controlled by Dox. Thus, there is no visible marker (i.e., GFP) for tracking initial lentiviral production and later target-cell transduction before Dox induction. Second, the gRNA or multiple gRNAs are on a separate vector, which increases the difficulty of cotransducing target cells. Third, the constitutive promoter used IRES linker for neomycin selective marker in this system reducing the selective strength of neomycin because the bulky IRES decreases its downstream gene expression.

Other inducible lentiviral Cas9 systems

There are several other inducible lentiviral Cas9 systems reported to date. For example, researchers constructed a 4-hydroxytamoxifen-inducible Cas9 by fusing a hormone-binding domain of the estrogen receptor ERT2 to Cas9 [19]. Also, investigators constructed a light-activated Cas9 by fusing a light-responsive element to Cas9 [20]. In crop research, a plant heat shock protein 17.5E (HSP17.5E) gene promoter successfully induced Cas9 expression [21]. Furthermore, researchers used an inducible CRISPR/Cas9 system with a Met3 promoter to control the Ade gene in yeast *Candida glabrata* because Ade- colonies are pinkish in color, whereas Ade+ colonies are creamy white [22]. The techniques used in these Cas9 systems, although powerful and suitable for specific organisms, are not widely applicable to all eukaryotes or mammals included in current genome-editing studies.

gRNA design

There is no doubt about the importance of effective gRNA design in CRISPR/Cas 9 gene editing systems. One simple rule for gRNA design is to target the first exons to make sure that all potential alternative NHEJ repair products are frameshifted or inserted with a stop codon. In our studies, we empirically designed gRNAs in the first exons after ATG, the start codon, when the first exons were too short, we used the second exons for gRNA design. This approach worked well in several of our studies of genes of interest, such as RhoA, Gli1, Gal3, Yap1, Sox9, and Grk3. In our experience, the closer a gRNA is to the start

codon ATG, the more likely the whole gene of interest is to be completely disrupted [2].

Many online gRNA design platforms are available. We have used the gRNA design software from Massachusetts Institute of Technology and the one from the German Cancer Research Center ' s E-Crisp website. Both software are based on DNA sequences and the protospacer-adjacent motif (PAM, i.e. NGG), and the former also searched the whole genome to find potential mismatches.

In regard to CRISPR/Cas9 gRNA design rules for predicting gRNA activity and specificity, one study demonstrated that the most commonly used type II SpCas9 gRNA tools simply targeted an NGG motif (PAM) on basis of DNA sequences alone [23]. Early computational tools for target-site screening were programs that simply recognized the pattern and identified instances of this motif [24], which may have led to the selection of ineffective gRNAs. Off-target activity of Cas9 is known to be influenced by not only DNA sequences but also chromatin accessibility. Therefore, even currently available tools for designing gRNAs that depend only on DNA sequences cannot accurately predict gRNAs with precise and effective on-target activity.

Editing activity (focusing on on-target editing) and specificity (avoiding off-target editing) are decisive factors in the success of any application of CRISPR/Cas9 technology. After *in silico* testing several predicating models and web tools for 14 on-target software and 7 off-target software, Wang et al. (2020) [25] concluded that none of the models they tested were perfect. Problems they encountered included a unclear mechanism, data imbalance, data heterogeneity, insufficient training dataset, lack of generalization ability, and cross-species inefficiency. Therefore, they developed machine and deep learning-based algorithms to predict the on- and off-target activities of CRISPR gRNA. These algorithms may be the most effective and reliable methods of making such predictions [25].

Enhancing on-target specificity and reducing off-target effect of CRISPR/Cas9

Off-target effect of genomic editing refers to nonspecific genetic modifications, which are unintended point mutations, indels, inversions, and translocations [9,26-28]. In other words, Cas9's unintended cleavage of DNA at sites that do not match the gRNA is considered off-target editing. We previously reported off-target editing in RhoA gene in that a Y42C mutation in the gRNA5 binding site did not prevent Cas9 editing [2].

One study demonstrated that off-target cleavage of Cas9 gRNA may occur more than 50% of the time, which would indicate a major problem with this editing system [29]. Another study demonstrated that overexpressed Cas9 could tolerate up to five-basepair mismatches between gRNAs and targets [8]. Lin et al. (2014) [9] reported 15 off-target sites identified in 27 different single gRNAs, each off-target site harbored a single-base bulge and one- to three-basepair mismatches with the gRNA sequence, demonstrating that Cas9 cleavage occurs at a wide variety of off-target sites.

Chromatin immunoprecipitation sequencing (ChIP-seq) analysis revealed that activated Cas9 rarely cleaves off-target sites without matching gRNAs, whereas inactivated Cas9 binds to the genome at many sites [30]. Usually, gRNA is turned on by the human U6 RNA promoter, but a sequence-optimized U6:3 RNA promoter is more potent for on-target editing of germline and somatic genomes in fruit-flies with homologous direct repair (HDR) targeting the gene of interest, and offsets nick-based mutagenesis [31]. Other dramatic findings are that truncated gRNAs can reduce undesired off-target mutagenesis by more than 5,000-fold without compromising their on-target genome-editing efficiencies. The truncated gRNAs are shorter than 20 nucleotides and are at the opposite end of the PAM sequences [32,33]. In addition, a study comparing the activities of zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) to CRISPR/Cas9 method concluded that the latter yielded the least off-target editing [34].

Given these findings, researchers have concluded that overexpressed Cas9 is one of the factors causing off-target effects in the CRISPR/Cas9 system. A simple yet efficient strategy for controlling these off-target effects involves Cas9 self-targeting [35]. A more advanced method of overcoming off-target effects is the substitution of four amino acids in Cas9, this forms the " high-fidelity " Cas9 nuclease, SpCas9-HF1, which eradicates nearly all off-target events, as demonstrated by genome-wide sequencing [36]. Researchers developed another specificity-enhanced variant of Cas9, eSpCas9 from *Streptococcus pyogenes* to improve Cas9's specificity and reduce off-target cleavage while maintaining excellent on-target activity [37]. A new, highly accurate Cas9 variant that has high genome-wide specificity in human cells is HypaCas9, which investigators developed from the non-catalytic Cas9 domain, REC3 [38]. SaCas9-HF, a modified Cas9 variant from *Staphylococcus aureus*, has demonstrated high on-target accuracy [39]. Today, the most used Cas9 variant, also discovered in *S. aureus*, is SpCas9, but FnCas9 from the bacterium *Francisella novicida*, a SpCas9 homolog, has demonstrated higher specificity than SpCas9 [40]. In another report, a single point mutant of Cas9, R691A, was named HiFi-Cas9-R691A and abated off-target editing across the genome while maintaining high on-target specificity [41]. In comparison with eSpCas9 [37], SpCas9-HF1 [36], and HypaCas9 [38], HiFi-Cas9-R691A is more hopeful in future therapeutic applications.

We concluded that increasing Cas9's specificity, reducing its off-target editing by improving CRISPR/Cas9 technologies through molecular engineering, and discovering *de novo* Cas9- and Cas13-like genes in eubacteria, archaea, or other prokaryotes will be the foci for future gene mining and editing research. Progress in these areas is needed before CRISPR/Cas9 technology can be safely adopted for therapeutic applications.

RNA-editing technologies with CRISPR/Cas13, ADAR and APOBEC1

Over the past few years, RNA-editing technologies employing the CRISPR technology and nuclease Cas13, adenosine deaminase acting on RNA (ADAR), and apolipoprotein B mRNA-

editing enzyme, catalytic polypeptide 1 (APOBEC1) and their derived enzymes have become increasingly hot topics in gene editing, mRNA editing, and viral restriction [42](Figure 1B and 1C).

CRISPR/Cas13

In 2017, it was first reported that the RNA editing for programmable adenosine-to-inosine replacement (REPAIR) system, which uses Cas13 to edit RNAs, alters coding mRNAs [43]. Cas13 RNA editing provides another grand field of manipulations at molecular levels. Dr. F. Zhang's group at the Broad Institute, which has been a leader in making *de novo* discoveries in this RNA-editing field, believes that nucleic acid editing at the RNA level holds even more promises than genome editing as a means of treating genetic diseases, particularly in cases in which disease-relevant sequences can be rescued to yield functional protein products [10]. Currently, two CRISPR/Cas13 systems are available: type VI-A/C and type VI-B, both of which employ similar DNA structures.

The RNA-editing system of CRISPR/Cas13 has multiple advantages over the DNA-editing system of CRISPR/Cas9 First, it works in non-dividing cells. Second, Cas13 enzymes do not need a PAM sequence at the target locus. Third, some Cas13 enzymes prefer targets with a single-base protospacer flanking site (PFS) sequence, but the ortholog LwaCas13a does not require a specific sequence. Fourth, Cas13 enzymes do not directly edit the genome. Therefore Cas13-based RNA-editing systems are more likely to produce reversible results than genome editing and to prevent genomic off-target activities and indels introduced by Cas9. Studies demonstrated that ADARs, which are enzymes that can convert the nucleotide adenosine to inosine (Figure 1B), can be fused with Cas13 to markedly improve CRISPR/Cas13's RNA-editing capability [10]. However, recent studies of Cas13 have focused on CRISPR/Cas13 ' s structure rather than its applications in RNA editing.

Researchers have used rewired CRISPR/Cas9 and CRISPR/Cas13 systems to combat viruses in eukaryotes due to their targeting of DNA and RNA viruses, respectively [44]. DNA viruses include herpes viruses, papillomaviruses, and hepatitis B virus; whereas RNA viruses include the human immunodeficiency virus (HIV), which involves the reverse transcription of RNA genomes into double-stranded DNA proviruses, and some deadly coronaviruses, such as those viruses of severe acute respiratory syndrome SARS-coV and SARS-coV2 (COVID-19), and Middle East respiratory syndrome MERS-coV.

ADAR, REPAIR, and RESCUE

ADAR is an enzyme that is capable to convert a nucleotide adenosine-to-inosine (A?I) (Figure 1B). The ADAR gene is located on chromosome 1 in humans, which is unrelated to eubacterial and archaeal immunity genes, such as Cas9 and Cas13. The programmable, two-component REPAIR system consists of an inactivated Cas13 enzyme (dCas13) fused to an ADAR (such as ADAR2). Using the REPAIR technique, Cox et al. (2017) [10] successfully edited 33 of 34 sites in RNAs of HEK293T cells with a maximum of 28% editing efficiency as assessed using RNAseq.

However, like Cas9, ADARs and other site-directed RNA-editing enzymes suffer abundant off-target edits, although they can be reduced via nuclear localization of the enzyme [45]. In a study by Cox et al. (2017) [10], dCas13 (inactive)/ADAR2 demonstrated robust knockdown of RNA transcripts, but ADARDD (ADAR2 deaminase domain with the E488Q mutation) also caused a substantial number of off-target edits in KRAS gene, as demonstrated by transcriptome-wide analysis .

Another RNA-editing system, the RNA editing for specific C-to-U exchange (RESCUE) system directly evolves ADAR2 into a cytidine deaminase. RESCUE doubles the number of mutations targeted by RNA editing and enables modulation of phosphosignaling-relevant residues. It retains adenosine-to-inosine editing activity, enabling multiplexed cytidine-to-uridine and adenosine-to-inosine editing through the use of tailored gRNAs [46].

Both ADAR and REPAIR convert adenosine to inosine (to guanosine upon translation), leading to the potential for RNA mutations or restoration of dysfunctional proteins, although silent mutations may occur if the conversion is at the third position of a codon. Compared with ADAR and REPAIR, RESCUE has more promising applications as it works in both adenosine-to-inosine and cytidine-to-uridine conversions and therefore extends the reach of RNA editing. In addition, RESCUE may introduce a stop codon from CAA to UAA to disrupt target mRNA translation.

APOBEC1

APOBEC1 (Apolipoprotein B mRNA editing enzyme, also known as A1) is another cytidine-to-uridine editing enzyme. APOBEC1 has been linked with cholesterol control, cancer development, and inhibition of viral replication, and the RNA-editing is highly specific (Figure 1C). In the small intestine, for example, a cytidine in apolipoprotein B mRNA is converted to uridine, which in turn converts a glutamine (CAA) into a stop codon (UAA) to create a truncated apolipoprotein B [47]. APOBEC1 is unrelated to eubacterial and archaeal immunity genes, such as Cas9 and Cas13 as well; instead, it is a human gene on chromosome 12. It is part of the vertebrate innate immune APOBEC family of genes, which play roles in viral restriction [48] and obviously evolved from a single pre-vertebrate APOBEC gene.

Unlike ADAR, REPAIR, and RESCUE, APOBEC1 is still in its early stages as an editing tool and needs further validation, improvement, and scrutiny. Although its conversion of codons from CAA to UAA signifies the introduction of a stop codon, any tweezing of a normal gene in the mammalian genome and transcriptome may have unintended consequences, since both ADAR and APOBEC1 are present in human chromosomes. Nucleases Cas9, Cas13, and even the common GFP and mOrange markers, and luciferase genes, are exogenous to mammals, their interaction with the mammalian cellular machinery is minimal at most.

Conclusion and prospects

The discovery of CRISPR/Cas technologies has led to breakthroughs in molecular and cellular biology over the past decades. These technologies have been the most popular systems for gene disruption, mutation, and insertion via cellular NHEJ and HDR repairs. In addition, LentiCrisprV2 is the most popular gene-editing and KO tool used in research labs today, and researchers have made many improvements and modifications to this easy-to-use, efficient transformation and transduction system.

Herein, we described our improved lentiviral vector, V2mO, and our addition of a visual marker for cell sorting and monitoring lentiviral production and transduction efficiencies. We also described other improvements to the CRISPR/Cas9 system, including methods of directly estimating the gene-editing efficiency of target cell pools and rescuing genes by overcoming Cas9 editing. In addition, we provide information on several Dox-inducible Cas9 systems, including TLCV2 (Figure 3A) and Lenti-iCas9-Neo (Figure 3B). These inducible systems are advantageous for studying the downstream effects of essential genes because they can prevent cell death before Dox-induced Cas9 activation and essential gene disruption. Another advantage of inducible systems is that low levels of Dox induction may abate off-target editing caused by Cas9 overexpression.

Over the past few years, use of RNA-editing technologies, such as ADAR, REPAIR, RESCUE, and APOBEC1, has gained widespread attention. APOBEC1 ' s surprising RNA-editing capability is apparent even in vertebrates, and novel mechanisms of RNA-editing are still being discovered. RNA-editing techniques for either the conversion of a single nucleotide or the introduction of a new stop codon without genomic alteration are also promising for both basic research and clinical applications due to their decreased off-target effects and ability to reverse gene function. However, RNA-editing systems need further explorations and improvements via molecular engineering and de novo discoveries into known and unknown prokaryotes and eukaryotes.

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Conflict of interest

The authors declare there is no conflict of interest.

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