The steady progress in genome editing, especially genome editing based on the use of clustered regularly interspaced short palindromic repeats (CRISPR) and programmable nucleases to make precise modifications to genetic material, has provided enormous opportunities to advance biomedical research and promote human health. The application of these technologies in basic biomedical research has yielded significant advances in identifying and studying key molecular targets relevant to human diseases and their treatment. The clinical translation of genome editing techniques offers unprecedented biomedical engineering capabilities in the diagnosis, prevention, and treatment of disease or disability. Here, we provide a general summary of emerging biomedical applications of genome editing, including open challenges. We also summarize the tools of genome editing and the insights derived from their applications, hoping to accelerate new discoveries and therapies in biomedicine.

gene editing, CRISPR, high-throughput functional genomics, diagnostics, animal model, therapeutics
Introduction

The purpose of gene-editing technology is to precisely change DNA sequences at target sites. By fusing zinc finger (ZF) proteins or transcription activator-like effector proteins with the cleavage domain of FokI endonuclease, zinc finger nucleases (ZFNs) (Beerli and Barbas, 2002) or transcription activator-like effector nucleases (TALENs) (Boch and Bonnas, 2010) were developed, respectively, which started the era of programmable gene editing. More recently, under the direction of a guide RNA (gRNA), clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) nuclease can cleave the DNA double-strand at target sites with great convenience, efficiency, and precision (Chang et al., 2013; Cong et al., 2013; Jinek et al., 2012; Jinek et al., 2013; Mali et al., 2013).

Double-stranded breaks (DSBs) in genomic DNA are repaired by two endogenous pathways: nonhomologous end joining (NHEJ) and homology-directed repair (HDR) (Cecaldi et al., 2016). NHEJ introduces random insertions or deletions (indels) of nucleotides, which often lead to open reading frame shift mutations (Deriano and Roth, 2013) and ultimately disrupt the expression of the target gene (so-called “knockout”). Alternatively, precise sequence replacement or insertion (so-called “knockin”) can be achieved via HDR when donor DNA is present (Jasin and Rothstein, 2013). However, HDR efficiency is normally low in many types of cells, limiting the breadth of its applications in biomedical research and gene therapy. Recently, by fusing nuclease activity-impaired Cas proteins with different effector modules, including nucleobase deaminase and reverse transcriptase, base editors (BEs) (Gaudelli et al., 2017; Komor et al., 2016) and prime editors (PEs) (Anzalone et al., 2019) have been developed to achieve precise editing with high efficiency and product purity.

This section reviews the development and improvement of various gene-editing technologies, including ZFN, TALEN, CRISPR/Cas, BE, PE, and RNA editing (Table 1).

Table 1  Representative editors

| Genome editor | Targeting ability | Knock out | Knock in | Base substitution | Off-target effects | Reference |
|---------------|-------------------|-----------|----------|-------------------|-------------------|-----------|
| ZFN           | +                 | +++       | +        | +                 | +                 | (Bibikova et al., 2002; Kim et al., 1996) |
| TALEN         | ++                | +++       | +        | +                 | +++               | (Cermak et al., 2011; Christian et al., 2010; Miller et al., 2011) |
| Cas9          | +++ (NGG PAM)     | +++       | +        | +                 | +++               | (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013) |
| Cas12a        | +++ (NGG PAM)     | +++       | +        | +                 | ++                | (Zetsche et al., 2015) |
| nCas9         | ++ (sgRNA pair, NGG PAM) | ++ | +        | +                 | ++                | (Ran et al., 2013; Shen et al., 2014) |
| dCas9-FokI    | ++ (sgRNA pair, NGG PAM) | ++ | +        | +                 | +                 | (Guilinger et al., 2014; Tsai et al., 2014) |
| BE3           | ++ (NGG PAM)      | +++       | (Creating stop codon) | −                 | +++ (C-to-T/G-to-A) | +++ (Komor et al., 2016) |
| hA3A-BE3      | ++ (NGG PAM)      | +++       | (Creating stop codon) | −                 | +++ (C-to-T/G-to-A) | +++ (Wang et al., 2018e) |
| dCas12a-BE    | ++ (TTTV PAM)     | +++       | (Creating stop codon) | −                 | +++ (C-to-T/G-to-A) | + (Li et al., 2018d) |
| BEACON        | ++ (TTTV PAM)     | +++       | (Creating stop codon) | −                 | +++ (C-to-T/G-to-A) | + (Wang et al., 2020f) |
| tBE           | ++ (NGG or NG PAM) | +++       | (Creating stop codon) | −                 | +++ (C-to-T/G-to-A) | − (Wang et al., 2021) |
| ABE7.10       | ++ (NGG PAM)      | +++       | (Mutating splicing site) | −                 | +++ (A-to-G/T-to-C) | +++ (Gaudelli et al., 2017) |
| LbABE8e       | ++ (TTTV PAM)     | +++       | (Mutating splicing site) | −                 | +++ (A-to-G/T-to-C) | ++ (Richter et al., 2020) |
| PE3           | ++ (NGG PAM)      | +++       | (Creating stop codon) | (Small insertion) | ++                 | +/- (Anzalone et al., 2019) |
| Cas13         | +++ (RNA, no PFS constraint) | ++ (Knock down) | −       | −                 | ++                 | (Abudayyeh et al., 2017) |
| REPAIR        | ++ (RNA, no PFS constraint) | + (Knock down, mutating start codon) | −      | ++ (A-to-G/T-to-C) | ++                 | (Cox et al., 2017) |
| RESCUE        | ++ (RNA, no PFS constraint) | ++ (Knock down, creating stop codon) | −      | ++ (C-to-T/G-to-A) | ++                 | (Abudayyeh et al., 2019) |

a) PFS: protospacer flanking site.
**ZFN**

Site-specific nucleases have long been applied in DNA recombination in vitro, and therefore, these nucleases were first used for gene editing. Meganucleases, a type of endonuclease that recognizes long DNA sequences (e.g., ~12–40 bp), have been applied and engineered to generate DSBs at genomic loci. However, meganucleases have not been used widely in genome editing due to their limited recognition sites and the difficulty of designing their targeting specificity. However, DNA endonucleases can be used as effector modules to generate DNA breaks. The FokI endonuclease in particular has separate domains responsible for DNA binding and cleavage. A single DNA cleavage domain of FokI is inactive, but the dimerization of two cleavage domains gains DNA cleavage activity and cuts DNA double strands with no sequence specificity. Thus, a pair of fusion proteins, each containing a FokI DNA cleavage domain and a locator module, can be used for targeted gene editing.

Zinc finger motifs, originally discovered in transcription factors in *Xenopus laevis* (Klug, 2010; Miller et al., 1985), were used as the first locators for DNA targeting in gene editing. One ZF motif binds to three base pairs (bp) and an array of ZF motifs that recognize ~9–18 bp of specific DNA sequences (Bibikova et al., 2002). This modular configuration makes ZF a potential platform for programmable genome targeting. By fusing an array of ZF motifs with the FokI cleavage domain, ZFNs were developed for programmable gene editing (Kim et al., 1996). Generally, a pair of ZFNs that target the upstream and downstream regions of an intended genomic locus can be used to induce the dimerization of FokI cleavage domains, which then cleave DNA double strands. The repair of DSBs by NHEJ or HDR can eventually lead to random indels or precise sequence replacement for gene knockout or knockin, respectively.

Although some customized ZF arrays can efficiently bind to targeted genomic loci, the construction of ZF arrays for most genomic sites remains challenging, as the crosstalk between adjacent ZF motifs can interfere with the binding of a ZF array to the intended DNA region. Thus, the generation of a pair of functional ZFNs requires the screening of numerous ZF arrays. As a potential gene therapy tool, ZFNs have relatively small sizes and can be packaged into adeno-associated virus (AAV) (Yin et al., 2017a), a convenient and prevalent vector for in vivo gene editing.

**TALEN**

Discovered in the bacterial plant pathogen *Xanthomonas*, transcription activator-like effector (TALE) proteins contain DNA binding domains that are repeats of amino acid residues (Boch and Bonas, 2010). The DNA binding domain of TALE has tandem 33–34 amino acid repeats with divergent dual residues at positions 12 and 13. These two positions (the so-called repeat variable diresidue, RVD) are highly variable and determine the DNA binding specificity of a TALE protein (Boch et al., 2009; Moscou and Bogdanove, 2009). As a TALE motif containing a specific RVD can recognize a specific nucleotide, a combination of repeat TALE motifs containing the appropriate RVDs can bind to a specific DNA sequence. In contrast to ZF arrays, TALE arrays can recognize and bind to target sites without interfering with each TALE domain in the array.

Similar to ZFN, by taking advantage of a pair of fusion proteins of the FokI cleavage domain and TALE array, TALENs were developed to induce DSBs at targeted genomic sites (Cermak et al., 2011; Miller et al., 2011). Although a pair of functional TALENs can be generated without tedious screening of TALE arrays, the construction of TALEN-expressing vectors is still complicated due to homologous recombination between repetitive TALE sequences. In addition, the complex design and construction of TALEN-expressing vectors are time-consuming and costly, which also hampers some of their potential applications, such as high-throughput assays. Although a study showed that the incidence of OT editing of TALEN in human stem cells was low (Veres et al., 2014), the systematic analysis of OT effects by TALEN awaits further investigation.

**CRISPR-Cas**

CRISPR/Cas was originally identified as a defense system in bacteria to provide acquired immunity against bacterial parasites, such as bacteriophages and plasmids (Barrangou et al., 2007; Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Major types of CRISPR/Cas used for gene editing belong to the class 2 system (Makarova et al., 2020), which requires only one DNA endonuclease, e.g., Cas9 (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013) or Cas12a (also known as Cpf1) (Zetsche et al., 2015), to cleave double strands of bacteriophage or plasmid DNA under the guidance of CRISPR RNA (crRNA). In the CRISPR/Cas9 system, a crRNA and a trans-activating crRNA (tracrRNA) form a double-stranded RNA, which can be processed by RNase III, and then the mature crRNA/tracrRNA complex recruits Cas9 protein to form a ribonucleoprotein (RNP) complex by ZFNs in human cells, confirming the OT effects of ZFNs in vivo.
(Gasiunas et al., 2012; Jinek et al., 2012). Single-guide RNA (sgRNA) was engineered by fusing crRNA to scaffold tracrRNA to recruit Cas9 to facilitate gene editing in various species (Jinek et al., 2012). In the CRISPR/Cas12a system, only crRNA is needed to recruit the Cas12a protein to form an RNP complex (Zetsche et al., 2015). Directed by an sgRNA or a crRNA, the Cas9 or Cas12a RNP complex binds to the target site, which is complementary to the spacer region of the corresponding sgRNA or crRNA and has a protospacer-adjacent motif (PAM) (Anders et al., 2014; Nishimasu et al., 2014; Sternberg et al., 2014; Zetsche et al., 2015). Generally, different Cas proteins recognize different PAM sequences. For instance, the commonly used Streptococcus pyogenes Cas9 (SpCas9) recognizes an NGG PAM sequence at the 3’ end of the protospacer region (Jinek et al., 2012; Ran et al., 2015), while Acidaminococcus Cas12a (AsCas12a) and Lachnospiraceae bacterium Cas12a (LbCas12a) recognize a TTTV PAM sequence at the 5’ end of the protospacer region (Zetsche et al., 2015). After binding at a target site, the HNH and RuvC-like endonuclease domains of Cas9 cleave target (complementary) and non-target (noncomplementary) DNA strands, respectively (Jinek et al., 2012). The cleavage results in two blunt DNA ends, and the cleavage site is 3 bp upstream of the PAM sequence (Jinek et al., 2012). In contrast, Cas12a proteins have only a RuvC-like endonuclease domain, and they generate sticky DNA ends distal to the PAM sequence (Zetsche et al., 2015). The discovery of new Cas proteins and the engineering of discovered Cas proteins continuously expand the DNA targeting range of CRISPR/Cas systems (Hu et al., 2018; Kleinstiver et al., 2015; Miller et al., 2020; Nishimasu et al., 2018).

Genome-wide analyses have shown that Cas9 endonuclease can bind and cleave DNA double strands at OT sites with sequence similarity to the on-target sites (Kim et al., 2015; Tsai et al., 2015; Tsai et al., 2017). Generally, mismatches between the sgRNA spacer region and OT site can be better tolerated at the PAM-distal region than at the PAM-proximal region. Although the use of sgRNA with fewer potential OT sites in genomic DNA can reduce the OT effects of Cas9, the development of an improved CRISPR/Cas9 system with high editing specificity substantially reduced its OT effects. As Cas9 proteins contain two endonuclease domains, one endonuclease domain can be mutated, which results in a nickase version of Cas9 (nCas9). nCas9 can be used for gene knockout when it is co-expressed with a pair of sgRNAs targeting the opposite DNA strands of an on-target site (Ran et al., 2013). In this situation, nCas9 generates two nicks at opposite DNA strands, which mimics a DSB. In contrast, nCas9 generates only a DNA single-strand break (SSB) at a particular OT site as two sgRNAs have distinct OT sites, and thus the OT indels triggered by DSBs are largely avoided. However, SSBs can still induce some levels of indels at certain OT sites, as an SSB can be converted to a DSB through endogenous DNA repair processes involving endogenously expressed cytidine deaminases (Lei et al., 2018). The FokI cleavage domain was fused to catalytically dead Cas9 (dCas9) to decrease OT editing further. The fusion protein can induce DSBs at on-target sites where a pair of sgRNAs induce the dimerization and activation of FokI nuclease; however, no DNA break is generated at OT sites where the FokI cleavage domain remains inactive as a monomer (Fu et al., 2014; Guilinger et al., 2014).

Another strategy for improving editing specificity is to engineer Cas9 proteins. The residues of Cas9 that are involved in the interaction with the DNA backbone were mutated to reduce the binding of Cas9 at OT sites, while the binding and editing ability at on-target sites was largely retained (Chen et al., 2017a; Kleinstiver et al., 2016a; Lee et al., 2018; Slaymaker et al., 2016). In addition, sgRNAs for Cas9 have been modified to reduce OT effects, for instance, changing the length of the spacer region (Fu et al., 2014; Kim et al., 2015) or adding an RNA secondary structure onto the 5’ end of an sgRNA (Kocak et al., 2019). The method of delivery also affects the specificity of CRISPR/Cas9-mediated gene editing. Generally, the delivery of RNP complexes or RNAs provides higher editing specificity than DNA delivery, as the continuous expression of sgRNA and Cas9 from plasmid DNA can increase editing at OT sites (Kim et al., 2014; Ramakrishna et al., 2014; Rees et al., 2017; Yin et al., 2017b).

As ZFN, TALEN, and CRISPR/Cas all generate DSBs to initiate genome editing, the DNA damage response (DDR) triggered by DSBs has been observed in an increasing number of studies (Haapaniemi et al., 2018; Ihry et al., 2018). As a toxic DNA lesion, DSBs can trigger the phosphorylation and activation of a key DDR, ataxia-telangiectasia mutated (ATM) (Shiloh and Ziv, 2013). Furthermore, the end resection in the DSB repair process can generate single-stranded DNA regions, triggering the phosphorylation and activation of additional key DDR effectors, ATM and RAD3-related (ATR) protein kinase (Cimprich and Cortez, 2008). Both activated ATM and ATR can subsequently phosphorylate p53, resulting in cell cycle arrest or even cell death (Khanna et al., 1998; Tibbetts et al., 1999). Thus, preventing DDR would be a future direction to further improve gene-editing technology.

**Base editor**

The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC), and the activation-induced deaminase (AID) families of cytidine deaminases comprise various members in many species (Harris and Liddament, 2004; Salter et al., 2016; Yang et al., 2017). The APOBEC
family members can catalyze the deamination of cytidine to uracil in single-stranded nucleic acids, including RNA and single-stranded DNA (ssDNA). By fusing APOBECs with dCas9 or dCas12a proteins, cytosine base editors (CBEs) have been developed to induce C-to-U deamination in the ssDNA region of the R-loop generated by Cas9 or Cas12a (Hess et al., 2016; Komor et al., 2016; Li et al., 2018d; Ma et al., 2016; Nishida et al., 2016). To enhance editing efficiency, Komor et al. (2016) replaced dCas9 in CBE by nCas9 (D10A), which nicks the target strand and then triggers the endogenous mismatch repair (MMR) pathway (Kunkel and Erie, 2015). MMR resolves the CBE-induced U/G mismatch to a U/A pair by removing the unedited G-containing strand and then resynthesizes it complementary to the U-containing strand. Finally, the U/A base pair can be converted to a T/A base pair after DNA replication or repair. Although CBEs do not induce DSBs directly, the formation of indels was still found to be triggered by CBE because of the breakage of the abasic site that is formed after the excision of U by uracil DNA glycosylase (UDG) (Lei et al., 2018). Uracil DNA glycosylase inhibitor (UGI) was fused into or co-expressed with CBE to improve purity of the editing product and editing efficiency (Komor et al., 2017; Wang et al., 2017c).

As APOBEC family members can deaminate multiple cytidines in an ssDNA region, all the cytosines in the editing window (a few nucleotides in the spacer region) of CBEs can be edited, which hinders the application of CBEs when single-base editing is required. Thus, various mutations were introduced into the domains involved in the catalytic activity or substrate binding ability of APOBECs to reduce the cytidine deamination activities of CBEs and narrow their editing windows (Kim et al., 2017). In addition, DNA modification or dinucleotide sequence context, e.g., cytosine methylation or GpC context, also affects the editing efficiencies of CBEs. Hence, naturally occurring APOBECs or in vitro evolved APOBECs, including human APOBEC3A (hA3A) (Wang et al., 2018e) or evoAPOBEC1 (Thuronyi et al., 2019), have been used in CBEs to expand the editing scope.

Alternatively, *Escherichia coli* tRNA-specific adenosine deaminase (TadA), which catalyzes adenosine to inosine (I) deamination in tRNA, has been engineered to induce adenosine deamination in ssDNA (Gaudelli et al., 2017). Despite the originally low activity, TadA*, which was obtained after seven rounds of directed evolution in *vitro*, can successfully deaminate adenosine in DNA. To further improve the DNA binding activity of TadA*, wild-type TadA was fused at the N-terminus of TadA*; therefore, adenine base editors (ABEs) were developed by fusing nCas9 (D10A) with the TadA-TadA* heterodimer (Gaudelli et al., 2017). As inosines do not exist naturally in DNA, no known DNA glycosylase can efficiently remove inosines from deoxyribose. Thus, no DNA glycosylase inhibitor is required to be fused into ABEs, and no significant indel formation is triggered by ABEs. Similar to CBEs, subsequent MMR or DNA replication resolves the I/T mismatch to the I/C pair and eventually installs a G/C pair at the target site.

Recently, base editors were found to cause OT effects independent of sgRNA or Cas9. CBEs that contain APOBEC cytidine deaminases could induce genome-wide C-to-T/G-to-A mutations at OT sites with no sequence similarity to on-target sites, suggesting that the OT events are independent of the Cas9/sgRNA targeting module (Jin et al., 2019; Zuo et al., 2019). As APOBEC cytidine deaminases prefer ssDNA regions as deamination substrates, the APOBEC module of CBE can bind and trigger C-to-U deamination in ssDNA regions generated during various cellular processes, e.g., transcription, DNA replication, and repair (Chen et al., 2019b). Recently, by reducing the substrate-binding/catalytic activity of APOBEC or taking advantage of cytidine deaminase inhibitor domains, sgRNA-independent OT DNA editing was reduced or eliminated (Doman et al., 2020; Jin et al., 2020; Wang et al., 2021; Zuo et al., 2020).

Moreover, APOBEC1, the cytidine deaminase commonly used in base editors, was originally discovered to induce C-to-U editing in apolipoprotein B mRNA, and TadA, which evolved to perform A-to-G DNA editing, is an essential tRNA-specific adenosine deaminase in *E. coli*. Unexpectedly but not surprisingly, both CBEs and ABEs, which contain APOBEC cytidine deaminases and TadA* adenosine deaminases, respectively, induced transcriptome-wide C-to-U and A-to-I OT mutations (Grünewald et al., 2019a; Zhou et al., 2019a). By engineering the residues of APOBEC or TadA* involved in RNA binding, OT RNA editing was greatly reduced, and on-target DNA editing was maintained (Grünewald et al., 2019b; Zhou et al., 2019a). A recent review also summarized the progress to improve the editing efficiency and precision of CBEs and ABEs (Jeong et al., 2020).

**Prime editor**

Although CBEs and ABEs can efficiently induce C-to-U (G-to-A) and A-to-G (T-to-C) transitions, targeted transversions and precise small indels are still hard to generate, as the efficiency of HDR is generally low in most cells and tissues. A versatile gene-editing tool, prime editor (PE), has recently been developed to induce all twelve types of base substitutions, small indels, and their combinations with high efficiency and product purity (Anzalone et al., 2019; Yang et al., 2019). By conjugating nCas9 (H840A) with reverse transcriptase (RTase), the developed PE can initiate reverse transcription (RT) from the single-strand break generated in the nontarget strand under the direction of an engineered prime editing guide RNA (pegRNA). A pegRNA contains
three parts: a typical sgRNA containing a canonical spacer region for Cas9 recruitment and target-site binding, a primer binding site (PBS) to initiate RT, and an RT template to encode intended edits. Although the original PE induced only low levels of editing in mammalian cells, the editing efficiency was much improved by engineering Moloney murine leukemia virus (M-MLV) RTase to increase the binding ability at the RT initiation site, the thermostability, and the enzyme processivity. To further improve PE efficiency, a canonical sgRNA (nicking sgRNA) was also used to make a nick in the target strand, triggering downstream MMR to remove the unedited strand and maintain the edited strand. Moreover, by using a pair of pegRNAs that contain the same editing information but bind to opposite DNA strands, the efficiency of PE can be improved (Lin et al., 2021).

As the effector module of PE is an RTase from murine retrovirus, a recent study examined whether PE induces genome-wide mutations in plants. At gRNA-dependent OT sites, which have sequence similarity to on-target sites, PE induced minimal OT mutations (Kim et al., 2020). In addition, in contrast to BE, PE induced no observable gRNA-independent OT mutations throughout the genome of plant cells, demonstrating its high editing specificity (Jin et al., 2021).

RNA editing

In addition to targeting DNA, some class 2 CRISPR/Cas systems can also target RNA. Under the guidance of a single crRNA, Cas13 family members can bind to RNA with a corresponding target sequence, providing a platform for targeted RNA editing (Abudayyeh et al., 2016; Abudayyeh et al., 2017; Konermann et al., 2018; Xu et al., 2021a). Unlike DNA editing, RNA editing does not permanently change genetic information, thus having complementary strengths, e.g., applications in disease treatment without the risk of irreversible side effects. RNA-targeting Cas13 proteins have been fused with wild-type adenosine deaminase acting on RNA (ADAR) to induce A-to-I base editing in RNA (Cox et al., 2017). Alternatively, ADAR has evolved to deaminate cytidine in RNA and can be used to perform targeted C-to-U RNA base editing (Abudayyeh et al., 2019). Although RNA editing demonstrated considerable efficiency, its OT effects await systematic analysis. Surprisingly, native ADAR can also be recruited by engineered RNAs to perform A-to-I editing at target RNA in the absence of Cas13 protein (Merkle et al., 2019; Qu et al., 2019), which showed low OT effects.

High-throughput functional genomics

Cutting-edge genome editing technologies enable genomic alterations both individually and in a high-throughput fashion. CRISPR-Cas9 genomic screens drive the latest exciting biological findings, significantly advance the scope and accuracy of functional genomics and have outperformed RNA interference (RNAi) platform, owing to their robustness and scalability (Shalem et al., 2015).

CRISPR screen pipelines and strategies

Upon Cas9-sgRNA ribonucleoprotein targeting, the RuvC and HNH nuclease domains of Cas9 induce DSBs (Jinek et al., 2012). In response to nuclease-induced DSBs, random indels at the site of DNA cleavage are introduced by the cellular DNA repair system. These indels are vital to generating knockout phenotypes of coding genes.

Selecting active sgRNAs that mediate high CRISPR/Cas9 activity is critical to implement CRISPR genetic screens. The genome-wide selection of sgRNAs can be optimized in a systematic approach; for example, an effective library was created for gene repression and activation screens by applying an algorithm that incorporates chromatin, position, and sequence features (Horlbeck et al., 2016). Multiple sgRNAs targeting the same gene are generally recommended to increase the probability of editing and the robustness of deconvolution. The negative control and nontargeting sgRNAs are critical for quality control of screening and data interpretation. The cell coverage of sgRNAs is another important parameter for library performance.

A robust readout is decisive for a successful screen. The readout can be generalized into two categories (Figure 1). Cells are subjected to universal conditions, as cell growth or death occurs during selection, and the resultant cells can be collected as a whole population. Otherwise, populations of interest have to be enriched by various methods, including biological assays evaluating cell migration, cell attachment, and cellular fluorescence intensity.

Typical examples include screens to identify coding genes conferring resistance to a drug, toxin, pathogen, or immune cells (Guo et al., 2022; Liang et al., 2021; Peng et al., 2015; Ren et al., 2015; Shalem et al., 2014; Zhao et al., 2019; Zhou et al., 2014; Zhu et al., 2021). In a positive screen, the majority of cells are depleted under strong selection conditions, and only a few cells with a protective phenotype expand. In a negative screen, cell growth in a fixed condition and time period can be applied to identify genes whose perturbations lead to cell death or growth inhibition (Shalem et al., 2014; Wang et al., 2014). Fluorescence-activated cell sorting (FACS) is another frequently used assay to enrich or deplete populations of interest in a flexible manner. For example, the surface expression density of a given molecule can be used to enrich cells through FACS. Coding genes regulating immune molecule expression, such as Foxp3, Fas, PD1, and HLA, have been investigated (Burr et al., 2017; Cortez et al., 2020;
Dersh et al., 2021; Jiang et al., 2019).

Proficient molecular engineers have created Cas9 variants with different nuclease activities, PAM compatibilities, editing windows, and small-molecule or light dependences (Anzalone et al., 2020). Catalytically inactive Cas9 protein (dead Cas9, dCas9) binds a targeted DNA sequence but does not cleave the DNA strands (Pickar-Oliver and Gersbach, 2019). This feature was leveraged to generate Cas9 derivatives by coupling dCas9 with various effectors, such as transcriptional modulators (Gilbert et al., 2013; Qi et al., 2013) or epigenome-modifying factors (Liu et al., 2016). dCas9 transcriptional modulators have been successfully applied to achieve the transcriptional activation (CRISPRa) and repression (CRISPRi) of a gene. They were first established by fusing the transcription activator Vps64 or the Krüppel-associated box (KRAB) repressor to dCas9 (Gilbert et al., 2013). Endeavors have been made to improve the efficacy of these effectors, e.g., CRISPRa effectors were further improved by fusing a repeating peptide array of epitopes to recruit Vps64 (Tanenbaum et al., 2014) or using a synthetic sgRNA scaffold with an MS2 RNA motif loop to recruit additional activators (Joung et al., 2017). These Cas9 derivatives leading to gene transcriptional activation or repression have been employed in functional screens, and the latter type is particularly useful when gene transcriptional perturbation, rather than gene knockout, is preferred. These screens have identified coding genes that modify cell growth and confer cancer therapeutic resistance (Joung et al., 2017; Liu et al., 2016). dCas9-engineered epigenetic effectors have been summarized elsewhere (Nakamura et al., 2021). Notably, dCas9-coupled gene-regulatory effectors and epigenetic effectors have considerable overlap of gene regulation mechanisms. For instance, transcriptional modulators could shape epigenetic states, and vice versa. Recently, an effector (CRISPRoff) was generated by fusing the transcriptional modulator ZNF10-KRAB and epigenetic effector domains to dCas9 (Nuñez et al., 2021). This effector could silence the specific gene that is stably maintained across cell division and differentiation. All these approaches have demonstrated efficacy in diverse high-throughput genomic screens.

**Interrogating noncoding genes**

The CRISPR/Cas9 system offers a general platform for RNA-guided DNA targeting, including coding and noncoding genes. The CRISPR screening approach could be readily extended to systematically discover the functions of numerous noncoding transcripts. MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression posttranslationally. Small nucleolar RNAs (snoRNAs) constitute a group of intron-encoded noncoding RNAs. CRISPR/Cas9 knockout screens have revealed that multiple miRNAs and snoRNAs regulate cancer cell growth (Cui et al., 2021; Kurata and Lin, 2018; Wallace et al., 2016).

Long noncoding RNAs (lncRNAs) are particularly interesting because of their large numbers and expanding roles in a wide array of cellular processes. Although CRISPR/Cas9 is ideal for inducing frameshift mutations in exons of coding genes to achieve gene knockout, frameshifts are usually not sufficient to disrupt the structure and function of lncRNAs.
To tackle this, studies have applied large-fragment deletions and splice-site disruption strategies mediated by paired sgRNAs and a single sgRNA, respectively, to disrupt the expression of lncRNAs (Horlbeck et al., 2020; Liu et al., 2018a; Liu et al., 2020; Zhu et al., 2016). These studies have revealed a number of lncRNAs that affect human cancer cell fitness in a cell type-dependent manner. Transcriptional and epigenetic effectors can perturb the expression levels of genes, which is particularly helpful to study the function of lncRNAs. The first CRISPRi screen applied dCas9-KRAB targeting to 16,401 lncRNA loci to evaluate cellular growth in 7 diverse cell lines (Liu et al., 2017a). The same strategy has identified PRANCR as a novel regulator of epidermal homeostasis (Cai et al., 2020). Another study developed a genome-scale CRISPRa screen targeting more than 10,000 lncRNA transcriptional start sites to identify noncoding loci that confer cancer therapeutic resistance (Jounge et al., 2017).

Circular RNA (circRNA) is another class of noncoding RNA that features a covalent bond linking the 3’ and 5’ ends generated by backsplicing (Ebbesen et al., 2016). Increasing evidence suggests its role in the regulatory network governing gene expression. Given the identical sequence of circRNAs and their parental mRNA, targeting circRNA-forming exons or the intronic cis-elements required for circRNA biogenesis may affect parental gene expression. To specifically perturb the expression of circRNA, a recent study utilized CRISPR-RfxCas13d and gRNAs targeting the RNA sequences spanning back-splicing junction (BSJ) sites. Taking advantage of the low biogenesis efficiencies and turnover rates of circRNAs, this method interferes with circRNA expression more efficiently than linear mRNA and consequently identifies multiple circRNAs contributing to cell proliferation in a cell type-dependent manner (Li et al., 2021c).

**Mapping regulatory elements**

Proximal and distal enhancers are vital genomic elements dictating gene expression and cell programming. Various biochemical markers and chromatin features are widely used to predict regulatory elements, and reporter assays are applied to assess their effects on gene expression. The CRISPR/Cas9 system now enables researchers to functionally map these elements in a native genomic context through mutation or through transcriptional and epigenetic modulation.

A study was conducted to target predicted enhancer sites of p53 and ERα with CRISPR-Cas9 in a high-throughput fashion (Korkmaz et al., 2016) and revealed multiple novel functional enhancers. The same strategy was applied to identify CTCF-binding elements (CBEs) that are essential for ERα-driven cell proliferation (Korkmaz et al., 2019).

To finely map functional sites in specific enhancers, one study applied tiling libraries to generate saturation mutagenesis in situ at 3 DNase I hypersensitive sites (DHSs) of a BCL11A composite enhancer (Canver et al., 2015). Given that BCL11A abundance is inversely correlated with HbF protein expression, by the association of HbF levels and enriched sgRNAs, the study provided a nucleotide resolution map of BCL11A enhancers. The same approach has been applied to map functional sites in regulatory elements of CDKN1A (Korkmaz et al., 2016), NF1, NF2, CUL3, POU5F1 (Diao et al., 2016), Tdgf1, and Zfp42 (Rajagopal et al., 2016). The above studies applied fluorescence tags, surface protein staining, or assay-specific selection reagents to enrich cells of interest. An alternative approach to perform a tiling mutation screen is to apply paired deletion guides in close proximity to genomic loci of interest, including POU5F1 and HPRT1 (Diao et al., 2017; Gasperini et al., 2017).

Base editing strategies apply additional effectors, such as activation-induced cytidine deaminase (AID) (Nishida et al., 2016), APOBEC cytidine deaminases, and TadA adenine deaminase, in combination with the CRISPR-Cas9 gene-editing system to introduce point mutations at the targeted site (Gaudelli et al., 2017; Komor et al., 2016). A recent study applied an APOBECB-mediated base editor screen to map regulatory elements of four loci involved in HbF expression. A total of 6,174 sgRNAs targeting 307 putative regulatory elements were investigated by correlating enriched sgRNAs to HbF expression abundance, revealing novel therapeutic candidates for sickle cell disease treatment (Cheng et al., 2021).

dCas9 coupled with transcriptional and epigenetic effectors has also been applied to investigate distal regulatory elements. A study applied dCas9-KRAB repressor to assess ~1.3 Mb of genomic sequence surrounding two loci, GATA1 and MYC (Fulco et al., 2016). Multiple distal enhancers contributing to their expression were identified. Another study applied a dCas9-VP64 activator with a tiled library of gRNA targeting sites in the vicinity of CD69 and IL2RA to identify stimulation-responsive enhancers in T cells (Simeonov et al., 2017). Moreover, a study applied dCas9-KRAB (CRISPRa) and dCas-9p300 (CRISPRi) as effectors to investigate the enhancers of β-globin and HER2 (Klann et al., 2017). The above studies demonstrated that CRISPR/Cas9-based transcriptional and epigenetic screens are successful in identifying genomic regulatory elements.

**Probing functional residues**

Tiling libraries generating saturation mutagenesis in situ can also be applied to study functional residues of coding genes. Parsing fragmented DNA Sequences from CRISPR Tiling MuTagenesis Screening (PASTMUS) is a method pairing tiling mutagenesis and NGS to identify functionally critical
amino acids (Zhang et al., 2019c). Similar tiling mutagenesis using a cytosine base editor to identify functional residues of the protein of interest has also been reported. This method, called CRISPR-X, identified novel mutations that modulate protein expression and confer drug resistance (Hess et al., 2016). Recently, CBE has been applied to probe functional single-nucleotide variants in DDR genes, drug resistance, and cell growth under cellular stresses (Cuella-Martin et al., 2021; Hanna et al., 2021). These studies have demonstrated that base editors are effective in generating single nucleotide polymorphisms (SNPs) at endogenous loci.

Exploring genetic interactions

Given that most human diseases are caused by the combined action of more than one gene, high-throughput screens to illuminate gene interactions are in high demand. With improved dual-gene knockout gRNA vectors (Wong et al., 2016), CRISPR/Cas9-based screens might have been performed in synthetic-lethal studies (Han et al., 2017; Shen et al., 2017). It is still technically challenging to perform unbiased gene-interaction screens simply because of the immense number of pairwise combinations. To date, researchers have mainly focused on druggable and/or tumor inhibitory genes to identify synthetic lethal target pairs (Han et al., 2017; Shen et al., 2017).

Orthogonal CRISPR-Cas9 nucleases from _S. aureus_ and _S. pyogenes_ have been paired for synthetic lethal screening (Najm et al., 2018), which effectively reduced interference between delivered sgRNAs. Moreover, this combination can be further engineered to carry out a dual screen in which one gene is activated while another is inactivated in the same cell (Boettcher et al., 2018; Zhou and Wei, 2018).

Making better screens

In the majority of pooled screens, sgRNAs are integrated into the cellular genome for the purpose of decoding by NGS. Thus, libraries are normally introduced by retro- or lentiviral infection at low multiplicity of infection (MOI), usually <0.3, to ensure that most cells contain a single integrated sgRNA and thereby minimize the false-positive discovery rate (Shalem et al., 2014; Zhao et al., 2019; Zhou et al., 2014). For genome-wide screens at low MOI infections, a significantly large number of initial cells are required for library construction. A recent study established a strategy that enables infecting cells at a high MOI (Zhu et al., 2019). This assumes that both the false-positive and false-negative rates of screens could be significantly reduced with increasing replicates for each of the gRNAs. By engineering guide RNAs with multiple internal barcodes (iBARs), the enrichment of sgRNAs could be separately assessed with their associated barcodes. This has largely eliminated the codelivery of free riders with functional sgRNAs due to a high-MOI infection, significantly improving screening efficiency and accuracy in positive selection screens. However, the high level of cytotoxicity induced by multiple DSBs in high-MOI library construction hampered the application of the iBAR approach in negative selection screens. To resolve this issue, Xu et al. (2021c) reported a new genome-wide CRISPR screening method, termed iBARed cytosine base editing-mediated gene KO (BARBEKO). This new screening strategy uses CBE to generate gene knockout, thus eliminating DSB-related cytotoxicity.

High-throughput functional genomics can be further equipped with other technologies for a flexible readout. CRISPR screens could be combined with single-cell sequencing that profiles transcriptome or chromatin accessibility (Dixit et al., 2016; Jaitin et al., 2016; Rubin et al., 2019). These methods present various advances in detecting indirect or direct indices of sgRNA and linking rich information to single-cell identity (Jaitin et al., 2016; Mimitou et al., 2019; Replogle et al., 2020; Rubin et al., 2019). Combining gene perturbation and phenotypical information offers a great opportunity to tackle the complexity of the biological system, analyze genetic interactions at scale, and dissect gene regulatory networks (Adamson et al., 2016; Norman et al., 2019).

Functional genomic screens in primary cells, organoids, and animals are expected to obtain more physiological insights and relevant therapeutic targets. Although wtCas9-based knockout screens are mostly used, DSB-induced cytotoxicity is prone to affect primary cells. A screening strategy to avoid DSBs is to introduce gene knockout by base editors (Billon et al., 2017) or other DSB-independent CRISPR derivatives. BARBEKO has been developed to avoid DSB-related disadvantages in comparison with wtCas9-mediated knockout fitness screens (Xu et al., 2021c). In particular, internal barcodes are integrated into sgRNAs to facilitate high MOI-lentivirus transduction in various types of cells.

For _in vivo_ screens, vectors with improved delivery efficacy, libraries that require fewer primary cells, and effectors that provide more relevant functional insights are in high demand. The _in vivo_ screens can be roughly divided into two conditions: transplantation-based _in vivo_ screening and direct _in vivo_ screening (Chow and Chen, 2018). In transplantation-based screens, sgRNA-containing tumor cells or activated immune cells are transferred to recipient animals and subjected to selection conditions (Chen et al., 2015a; Chen et al., 2021; Dong et al., 2019). Although this approach raises concerns about autochthonous microenvironment representation, the delivery efficacy is relatively high, and delivered sgRNA would not interfere with the rest of the
cells. Direct in vivo screening is more challenging regarding delivery efficacy, library size, and cell-type specificity. The hydrodynamic injection of sgRNA-containing plasmids can be applied to screen only a handful of genes in hepatocytes using a transposase system (Weber et al., 2015). Chen and colleagues established an in vivo direct screening method to investigate tumor suppressors by delivering a sgRNA library directly into the mouse brain or liver with an AAV vector (Chow et al., 2017; Wang et al., 2018a). These AAV-delivered sgRNAs are unable to integrate into the genome and have to be decoded by targeted-capture sequencing. They further generated AAV-sleeping beauty hybrid vectors for immune cell delivery in vitro, and these vectors can potentially be applied for direct in vivo screens (Ye et al., 2019).

**Perspective**

The CRISPR/Cas9 system has revolutionized high-throughput functional genomics. The platform continuously facilitates scientific inquiries and reveals exciting biological findings, including host-microbe interactions, the functionality of immune cells, and cancer treatment candidates. CRISPR/Cas9-based gene perturbations also advance our capacity to study the functions of coding and noncoding genomic elements.

Considering that not all CRISPR/Cas9 tools are compatible with high-throughput purposes, collective efforts to optimize the CRISPR/Cas9 system are highly appreciated. Systematic approaches to optimize the platform would be beneficial to all researchers. Beyond the optimization of libraries and Cas9 effector-related issues, the development of new readouts and friendly pipelines is expected, including bioinformatics-derived insights.

CRISPR/Cas9 knockout screens are widely used and exhibit high consistency between independent screens from different laboratories with similar experimental settings (Goh et al., 2021; Shalem et al., 2014). Despite this encouraging reproducibility, it should be kept in mind that not all significant factors can be identified using one system, relating to the efficacy of guides, the specificity of effectors, heterogeneity of gene expression in different cells, and experimental designs. Thus, applying different Cas9 effectors and experimental settings to study the same scientific question could be complementary to some prior studies.

**The application of gene editing in animal model construction**

An increasing number of pathogenic mutations have been identified in the postgenomic era. An animal model paves the way for studying the disease’s progression and finding new therapies in medicine. With the development of gene editing tools that can precisely edit target sites, it has become increasingly efficient to generate the desired animals. Hundreds of different kinds of genetically modified animals made with editing tools, especially CRISPR/Cas, have been reported. Here, we review the progress of animal models produced by ZFN, TALEN, and CRISPR/Cas. We will then briefly introduce some established models related to preventing human diseases and discuss the future development of animal models.

**Methods to produce the animal model**

RNAi and antisense oligonucleotides (ASOs) can knockdown target gene expression in animals, but neither allows the generation of stable gene knockouts. Homologous recombination (HR) in embryo stem cells is a conventional method to generate gene-editing animals, which requires more than one year to obtain the desired phenotypes. (Dow and Lowe, 2012). Gene-editing technologies significantly improve editing efficiency by more than two orders of magnitude by making DSBs (Rouet et al., 1994). The main process to generate genetically modified animals is to deliver the editing system into the target cells or tissues. For gene knockout via NHEJ, only an editing system was applied. For gene substitution or insertion via HDR, the repair donor should be delivered with the targeting system. The donor may be single-stranded oligodeoxynucleotides (ssODNs) or double-stranded DNA (dsDNA) (Zhang et al., 2021). The editing system could also be used in different formats, including Cas9-sgRNA RNP (Menchaca et al., 2020), RNA (Yang et al., 2013b), virus particles (Swiech et al., 2015), plasmids (Xue et al., 2014), cationic lipid nucleic acids (Zuris et al., 2015), and lipid nanoparticles (Musumuru et al., 2021). Because of the different applications and animal species, there are three main methods to generate genetically modified animals (Figure 2). We will summarize the progress for each method and discuss its advantage and disadvantage.

**Embryo microinjection**

Microinjection technology is more than 100 years old. Gene editing using embryo microinjection has been widely applied in many animal species, such as mice (Wang et al., 2013), rats (Ma et al., 2014), monkeys (Niu et al., 2014), pigs (Wang et al., 2015b; Wang et al., 2016; Zhou et al., 2016), goats (Wang et al., 2015a), sheep (Menchaca et al., 2020), zebrafish (Auer et al., 2014) and many other species. All three editing systems, especially CRISPR/Cas9, have been used for embryo microinjection (Martinez-Lage et al., 2017; Sato et al., 2016). The wide application of embryo microinjection is based on editing efficiency. For example, it takes only approximately six months to obtain the desired mouse, regardless of knockout (Li et al., 2013), knock-in (Gurumurthy
et al., 2019), or base editing (Ryu et al., 2018). With the development of instruments, it has become more convenient to handle embryo injection with square-wave electroporation, which does not need an experienced operation (Xu, 2019). Because of the heterogeneity of NHEJ, which may result in mosaics, desired animals may be obtained in the F1 generation, especially for gene knock-in. It has been reported that RAD51 delivery facilitates homozygous conversion, which may be applied to generate homozygous animals in F0 in the future (Wilde et al., 2021). For base editing, it is possible to obtain homozygous mutant animals because of the homogeneity and high efficiency of the editing results. Prime editing systems are opening new avenues for point mutation, deletion, and insertion (Anzalone et al., 2019). The improved version may help produce animal models with different kinds of mutations (Liu et al., 2021b). For some species, the embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), edited ESCs or iPSCs could be transferred into the blastula to obtain the desired animals. In such cases, the desired animal model should be produced only when the edited cells are germline cells.

Somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) provides another method to produce animals with a consistent genetic background. SCNT has been applied in more than 20 different species, including pigs, mice, monkeys (Liu et al., 2018b), and others. For gene editing animal models using SCNT, fetal fibroblasts are used to receive the editing system, and then the positive cell clone will be picked and used as the donor for SCNT. Pigs and monkeys are two important model animals in human medicine. Gene editing combined with SCNT has been achieved in both species (Liu et al., 2018b; Yan et al., 2018; Zhou et al., 2015). The advantage of SCNT is that the animals produced by this technology are consistent without mosaics (Ryczek et al., 2021). For large animals,
gene editing combined with SCNT has an advantage compared with embryo microinjection. The disadvantage of SCNT is abnormal development and poor efficiency. Some effective improvements will be useful for its application (Czernik et al., 2019).

**In situ injection**
The animal models produced by embryo microinjection and SCNT have modified genes in all tissues. For conditional editing animal models, the Cre model could be used, and it will take a long time to observe the phenotype. For some human diseases, such as cancer, tumor cells initiate in some tissues, not the whole body. In situ injection showed an advantage for this purpose. Gene editing in adult animals could be used to simulate the development of tumor cells (Zuckermann et al., 2015). Recently, different methods have been reported to study the tissue-specific functions of genes. One of them is to establish a model with Cas9 expression in a specific tissue, and then the sgRNA could be introduced into animals (Carroll et al., 2016). Another method is to inject Cas9 and sgRNA simultaneously (Zuckermann et al., 2015). The injection methods and the package of the editing system vary. Tail intravenous injection, tissue injection (Zuckermann et al., 2015; Zuris et al., 2015), and intraperitoneal injection (Carroll et al., 2016) have all been reported. There are several options for the delivery of the editing symptoms, such as adeno-associated virus (Lin et al., 2020), adeno-virus (Ding et al., 2014a), lipid nanoparticles (Musumuru et al., 2021), plasmid (Xue et al., 2014), and cationic lipid nucleic acid (Zuris et al., 2015). Furthermore, to accurately control gene expression after injection, some inducible elements are used, such as light (Konermann et al., 2013) and drugs (Dow et al., 2015). Somatic gene editing through tissue injection has been applied in several kinds of cancer (Maresch et al., 2016; Sánchez-Rivera et al., 2014; Zuckermann et al., 2015). Compared to embryo microinjection and SCNT, tissue injection requires less time to observe the desired phenotype, although the editing efficiency of this method should be improved.

**Animal models promote mechanistic studies and treatment of human diseases**
Gene editing technology greatly speeds up the production of animal models, which help to interrogate human diseases. Here, we summarize the progress on animal models depending on the disease categories. There are also animals that are used as tools for producing specific models, such as Cas9-expressing pigs (Wang et al., 2017b). Instead of listing all reported animal models made of editing technology, we focus on some representative cases of human diseases, especially large animals, e.g., nonhuman primates (Chen et al., 2016) and pigs (Ladowski et al., 2019).

**Cancer**
Cancer, as one of the most detrimental diseases involving multiple processes, is responsible for the loss of tremendous amounts of life every year. Suitable animal models may help to recapitulate the underlying mechanism of cancer development (Mao et al., 2016; Torres-Ruiz and Rodriguez-Perales, 2015). The gene-editing system makes it possible to generate multiple gene mutations and paves the way to interrogate the development and progression of cancers. Some animal models have been established to target the genes related to given cancers. Xue et al. (2014) used hydrodynamic injection to deliver a CRISPR plasmid to the liver in mice. Three genes, Pten, p53, and CTNNB1, were targeted. The editing efficiency for Pten was approximately 2.6%±1.4%. They found that a mutation of Pten would elevate Akt phosphorylation and lipid accumulation in hepatocytes. Platt et al. (2014) demonstrated that targeting KRAS, p53, and LKB1 in Cre-dependent Cas9 knock-in mice would lead to macroscopic tumors of adenocarcinoma pathology in lung adenocarcinoma. They used AAV as a donor to generate the KRASG12D mutation, and the efficiency was approximately 1.8% at nine weeks postdelivery. Maresh et al. (2016) delivered CRISPR/Cas9 targeting multiple genes to the pancreas of adult mice, and they observed that 54% of the mice developed pancreatic cancer within 24 weeks. There will be large animal models and potential target genes available in the future. Thus far, mice and zebrafish are the two main species that have been leveraged to produce models for cancer research (Li et al., 2021d). There will be more large animal studies and novel genes to be targeted in the future.

**Neurological disorders**
Neurological disorders are the leading cause of disability and greatly compromise the quality of life, such as Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and stroke (Chooeng et al., 2016). There are many mouse models for neurological disorders (Swiech et al., 2015; Tsuchiya et al., 2015). Because of the difference between human and rodent models, most neuroprotective therapies fail at the stage of translation despite the fact that the animal model plays a vital role in studying such diseases (Chesselet and Carmichael, 2012). The wide use of gene editing systems makes it convenient to simulate gene mutations in large animals, such as monkeys (Kang et al., 2019). Large animals may be more suitable for neurological disorders. Zhou et al. (2019b) generated the SHANK3-mutant macaques. The sgRNA targeted exon 21 of the SHANK3 gene, and the editing system was microinjected into the embryos. Finally, they obtained five live newborns, 2 of them did not have the wild-type genotype. The correct phenotype of animals exhibited sleep disturbances, motor deficits, and increased repetitive behaviors, which is consistent with autism.
spectrum disorder. Chen et al. reported that they generated TALEN-edited MECP2 mutant cynomolgus monkeys. They microinjected TALEN-RNA into embryos and obtained five female mutants. After a comprehensive analysis of the mutant monkeys, they found a series of physiological, behavioral, and structural abnormalities resembling clinical manifestations of Rett syndromes (Chen et al., 2017b; Novarino, 2017). Yan et al. (2018) used CRISPR/Cas9 and SCNT to establish a knockin pig model that expresses full-length mutant huntingtin. They transfected fetal pig fibroblast cells with Cas9, sgRNA and the donor. A total of 2,430 fetal pig fibroblast cell clones were picked, and 9 of them contained heterozygously expanded human HTT exon 1. One of these was chosen for SCNT. The mutant pigs showed consistent movement, behavioral abnormalities, and early death, which conformed to the phenotype of Huntington’s disease. Gene editing and SCNT are widely used in large animal models to avoid mosaics and heterogeneity (Cibelli and Gurdon, 2018; Cyranoski, 2018).

Cardiovascular disease
Cardiovascular disease (CVD) is the first-largest disease in terms of morbidity and mortality. There are several types of cardiovascular disease that are the leading causes of morbidity and mortality (Savoji et al., 2019). There is an urgent need to develop animal models for mechanistic studies and therapeutic evaluation. Many genes or single nucleotide polymorphisms are related to CVD, such as PCSK9 and APOE (Heianza and Qi, 2019). Animal models targeting different genes have been established (Carreras et al., 2019; Carroll et al., 2016; Chadwick et al., 2017; Ding et al., 2014a; Musunuru et al., 2021; Wang et al., 2018d; Yuan et al., 2018). Ding et al. (2014a) used adenovirus to deliver a CRISPR/Cas9 system targeting the Pcsk9 gene in mouse liver. The editing efficiency of Pcsk9 was as high as >50%. They found that the mouse had decreased plasma levels of PCSK9. Carreras et al. (2019) generated a liver-specific human PCSK9 knock-in mouse model. The human PCSK9 gene was inserted at the Rosa 26 locus. The positive ES clone was picked using neo and DTA elements. The mutant model showed a human-like hypercholesterolemia phenotype. Musunuru et al. (2021) used the base editor in living cynomolgus monkeys to target the PCSK9 gene and demonstrated that the monkeys had reduced blood levels of PCSK9 and low-density lipoprotein cholesterol. Carroll et al. (2016) generated cardiac-specific Cas9 transgenic mice and then used adeno-associated virus 9 to target Myh6. The mice displayed severe cardiomyopathy and loss of cardiac function. Considering the complexity of CVD, an increasing number of animal models that target different genes should be produced. Animal models will benefit drug screening (Savoji et al., 2019).

Xenotransplantation
Xenotransplantation provides an alternative method for alleviating the shortage of organs for human transplantation. Most recent xenotransplantation research has focused on genetically modified pigs. Niu et al. (2017) used CRISPR/Cas9 to inactive all the porcine endogenous retroviruses in pigs. The cell surface carbohydrate antigens in pigs preclude the success of porcine xenografts. Many studies have focused on the elimination of the surface carbohydrate antigens (Butler et al., 2016; Fischer et al., 2020; Fu et al., 2020; Ladowski et al., 2019; Ryczek et al., 2021). In the future, the “ideal” modified pig could be produced with advanced gene-editing technology for the benefit of patients.

Regeneration
Regeneration means the ability to replace or restore the injured body. The success of regeneration in mammals will greatly benefit patients. The mechanism of regeneration remains to be elucidated. Lizards, salamanders, and zebrafish show the ability to regenerate (Daponte et al., 2021). Modified animal models should be generated to uncover the mechanism of regeneration. Recently, genetically modified lizard and zebrafish have been reported (Auer et al., 2014; Rasys et al., 2019). In the future, with the help of gene editing, animal models may help to achieve regeneration in mammals.

Development
Some gene mutations result in aberrant development, such as adrenal hypoplasia congenita (AHC), which is caused by a DAX1 mutation. Kang et al. generated the DAX1 mutation in cynomolgus monkeys. These DAX1-deficient monkeys showed defects in adrenal gland development and abnormal testis architecture with small cords. The observed phenotype resembles the findings in human patients, demonstrating that the animal models were suitable for AHC. The SIRT6 gene encodes a longevity protein in rodents, and its function in primates remains unknown. Zhang et al. (2018c) produced the SIRT6 knockout in cynomolgus monkeys, which showed developmental retardation. These results may provide mechanistic insight into human perinatal lethality syndrome. African turquoise killifish is a naturally short-lived vertebrate and a good model to study aging. Harel et al. (2015) reported a platform that could generate genetically modified killifish for aging research. They acquired Tert mutant killifish using CRISPR/Cas9 and found that TertΔ8/Δ8 fish exhibited a progressive loss of fertility in the first generation.

Other Mendelian genetic diseases
There are many other disease-related animal models. Huang et al. (2019) produced HBB-deficient Macaca fascicularis monkeys manifesting severe β-thalassemia phenotypes,
which could be a valuable model for studying the mechanism of β-thalassemia and evaluating therapeutic interventions and drug effects. Chen et al. (2015b) reported the disruption of the dystrophin gene in rhesus monkeys and found that the mutant monkeys presented markedly depleted dystrophin and muscle degeneration. Lin et al. (2016) generated a nonhuman primate model by subretinal delivery of an AAV-mediated CRISPR-Cas9 system targeting CNGB3. The editing efficiency was 12%–14%. They observed a reduced response of electroretinogram in the central retina. Menchaca et al. (2020) produced a sheep model with mutations in the Otof gene. They used CRISPR/Cas9 combined with an ssODN to generate mutant lambs. A total of 17.8% of the lambs showed indels, and 61.5% of the edited lambs carried knock-in mutations. Large animals with disease-relevant mutation(s) may pave the way to treat malfunctions and facilitate successful translation from animals to humans.

**Perspective**

To date, mice are the most popular animals for simulating human diseases. Genome editing technology is opening a new avenue for efficiently producing animal models (Martinez-Lage et al., 2017). Considering the complexity of human diseases, it is pivotal to build suitable animal models. Recently, most genetically modified animals have been produced by knocking out target genes. In the future, animal models not only provide tools to understand disease mechanisms but are also valuable tools to evaluate therapeutics and clinical interventions, such as large animals and humanized animals.

Some characteristics of large animals, such as neuroanatomical, physiological, perceptual, and behavioral characteristics, are relatively similar to those of humans (Izpisua Belmonte et al., 2015). Large animals, such as chimpanzees, monkeys, pigs, and dogs, not only provide materials to accurately dissect disease mechanisms but are also suitable models to cure diseases, especially in the brain.

Previously reported animal models were produced mainly via gene knockout or point mutations. Base editing system and prime editing could efficiently achieve the base transition, transversion, insertion or deletion of a small fragment. It is still difficult to precisely handle large fragments. Few animals were obtained by replacement or knocking in the large fragment. Although some methods have been reported to insert large fragments, such as the INTEGRATE system (Vo et al., 2021), transposon-directed integration (Klompe et al., 2019; Strecker et al., 2019b) and CRISPR-Retron system (Sharon et al., 2018), their applications in mammals remain to be tested. The efficient generation of humanized animals, especially large animals, will be a future direction.

**Diagnosis**

**The development of CRISPR diagnosis**

In addition to its application in genome editing, CRISPR technology has recently been repurposed for the detection of target nucleic acids, among other tasks (Leung et al., 2021; Li et al., 2019b). Compared with traditional molecular diagnostic methods such as polymerase chain reaction (PCR), CRISPR-based diagnostic methods have shown many advantages in rapidness, accuracy, and portability and have been called next-generation molecular diagnostics (MDx) (Chertow, 2018). Based on the catalytic principles of the employed Cas proteins, the recently developed CRISPR diagnosis (CRISPR-Dx) systems can be simply classified into two categories (Figure 3). The first category employs Cas9 to detect target nucleic acids with the help of its high specificity in nucleic acid recognition. The second category uses Cas proteins with trans-cleavage activities, such as Cas12 and Cas13, and shows high specificity and sensitivity in detecting both nucleic acids and other molecules of interest (Li et al., 2019b).

**Cas9-based CRISPR-Dx**

The utilization of Cas9 and its mutants in nucleic acid detection undoubtedly constituted the prologue of CRISPR-Dx (Deng et al., 2015; Pardee et al., 2016). With the combination of an isothermal RNA amplification method called NASBA (nucleic acid sequence-based amplification), Cas9 was successfully used for pathogen diagnostics (Pardee et al., 2016). The obtained method, namely, NASBACC (NASBA-CRISPR Cleavage), takes advantage of Cas9-mediated precise cleavage and discriminates between distinct viral strains with single-base resolution. Specifically, target RNA is first amplified by NASBA, and the obtained amplicons are then digested with Cas9. If the target sequence perfectly matches the sgRNA, it will be cleaved by Cas9 and unable to trigger the coupled toehold RNA sensors. However, if the target sequence contains a mutation that prevents Cas9 cleavage, the intact amplicons are then transcribed to RNA sequences to switch on the sensor, resulting in a visible color change in the paper disc (Pardee et al., 2016). Moreover, to achieve high detection specificity in strain discrimination, strain-specific PAM sites can be employed for designing sgRNAs.

Since then, several CRISPR-Dx systems have been created by integrating Cas9 with different types of nucleic acid amplification methods, including nicking endonuclease-mediated nucleic acid amplification in CAS-EXPAR (Huang et al., 2018), rolling circle amplification (RCA) in CasPLA (Zhang et al., 2018b), RACE (Wang et al., 2020d), and the PCR method ctPCR (Wang et al., 2018c) and CARP (Zhang et al., 2018a). These methods mainly use Cas9-mediated precise cleavage activities and detect either fluorescent signals or amplicon sizes, all of which have shown both
sensitivity and specificity in nucleic acid detection. Another large group of detection methods employs dCas9, which has lost its nuclease activity, to specifically recognize and bind target nucleic acids. For example, the first CRISPR-based nucleic acid detection system, termed CASFISH (Cas9-mediated fluorescence in situ hybridization), employed fluorescently labeled dCas9 and sgRNA to precisely target and visualize genomic loci, revealing their great potential in genetic diagnosis (Deng et al., 2015). Recently, with the employment of sgRNAs linked with tandem MS2 aptamers and dCas9, a similar method, namely, RCasFISH (CRISPR/dCas9-MS2-based RNA fluorescence in situ hybridization assay), was developed to image and quantitate RNA transcripts at a single-molecule level (Wang et al., 2020c). Similar to Cas9, target nucleic acids can be pre-amplified to enhance the dCas9-based detection sensitivity. In addition to the fluorescently labeled probes, the coupled reporter can be split into two half domains, each half fused with an intact dCas9, and only when a pair of dCas9 binds to the target sequences in proximity can the split halves reconstitute an active reporter to produce signals. This strategy has been successfully employed in the development of PCR reporters (Zhang et al., 2017a) and RCH (Qiu et al., 2018), both of which are of high specificity due to the coexistence of two sgRNA binding sites as a prerequisite.

Scientists also use a Cas9 nickase (Cas9n) with only the functional RuvC domain to develop isothermal amplification systems for nucleic acid detection. For example, the CRISRDA method integrates Cas9n with the strand displacement amplification (SDA) method (Zhou et al., 2018), while the Cas9nAR method merely uses two enzymes of Cas9n and the Klenow polymerase (exo−) (Wang et al., 2019c), both of which exhibit high sensitivity and single-base resolution.

Other systems, such as CASLFA (Wang et al., 2020e), may use either Cas9 or dCas9 for the specific recognition of target sequences. CASLFA first uses either PCR or isothermal amplification for nucleic acid preamplification and takes advantage of the lateral flow assay (LFA) for convenient signal detection. Another promising CRISPR-Dx strategy is to apply a graphene-based field-effect transistor, which anchors the dCas9-sgRNA complex (dRNP) to a graphene monolayer and detects the on-chip electrical response after dRNP binds the target nucleic acid sequences. Notably, this method (namely, CRISPR-Chip) directly detects target sequences within 15 min, with a sensitivity of 1.7 fmol L⁻¹, and is amplification-free (Hajian et al., 2019). On the basis of CRISPR-Chip, the SNP-Chip is then created with the
employment of either Cas9 or dCas9 and discriminates between homozygous and heterozygous DNA sequences from unamplified patient samples (Balderston et al., 2021), demonstrating its great potential in diagnosis.

**Cas12-based CRISPR-Dx**

Cas9-based CRISPR-Dx technology has provided a new method for rapid nucleic acid detection. In addition, the discovery of the trans-cleavage activities of some Cas proteins has accelerated the development and application of CRISPR-Dx technologies, introducing the era of next-generation MDx. Usually, a Cas protein recognizes target nucleic acid sequences with the guidance of crRNA and cleaves the target sequences at specific sites, which is called cis-cleavage (Li et al., 2018b). While some Cas proteins exhibit nonspecific cleavage activities against nontarget single-stranded nucleic acids, these activities are designated trans-or collateral cleavage activities (Abudayyeh et al., 2016; Li et al., 2018b). To date, two types of Cas proteins have been demonstrated to possess trans-cleavage activities, including the type V Cas12-family and the type VI Cas13-family proteins (Li et al., 2019b).

The Cas12 protein recognizes and cis-cleaves the target dsDNA or ssDNA under the guidance of crRNA or sgRNA (Yang et al., 2016; Zetsche et al., 2015). Several Cas12 family members have been characterized to possess trans-cleavage activities, including Cas12a (previously called Cpf1), Cas12b (previously called C2c1), and Cas12F (previously called Cas14) (Chen et al., 2018; Harrington et al., 2018; Li et al., 2018b; Li et al., 2019a), and have been employed to develop diverse CRISPR-Dx systems. The trans-cleavage activities of Cas12a were first identified during the exploration of its cleavage activity against target ssDNA, and researchers found that once a ternary complex of Cas12a, crRNA and target dsDNA (or ssDNA) was formed, Cas12a was triggered to randomly cleave nontarget ssDNA in the system (Li et al., 2018b). Therefore, Cas12a has the properties of both target-specific binding and nonspecific ssDNA trans-cleavage and was used to create the CRISPR-Dx method, namely, HOLMES (a one-hour low-cost multipurpose highly efficient system) (Li et al., 2018c; Li et al., 2019b), which exhibits attomolar detection sensitivity and single-base resolution. Another group independently developed a similar Cas12a-based diagnostic method named DETECTR (Chen et al., 2018). Both methods employ Cas12a trans-cleavage activities to randomly cleave the fluorophore quencher (FQ)-labeled ssDNA reporter, and the only difference between them is that different pre-amplification methods are applied, i.e., PCR in HOLMES and RPA in DETECTR. Although the two methods were published almost at the same time, HOLMES was patented first and had a much earlier priority date than DETECTR (Wang et al., 2017a).

On the basis of HOLMES (or DETECTR), dozens of methods have recently been developed to further improve the diagnostic performance and practicability, including sample processing (Joung et al., 2020; Ning et al., 2021), target nucleic acid amplification (Aman et al., 2020; Ding et al., 2020; Lee et al., 2020), CRISPR reaction (Nguyen et al., 2020; Yue et al., 2021) and signal detection (Dai et al., 2019; Huang et al., 2020a; Nouri et al., 2020; Shao et al., 2019; Tao et al., 2020; Zhang et al., 2020). For example, nucleic acids can be released from direct sample lysis with an optimized extraction solution and concentrated by magnetic beads, eliminating tedious nucleic acid purification steps (Joung et al., 2020; Ning et al., 2021). In the original procedure of either HOLMES or DETECTR, the steps of nucleic acid amplification and CRISPR trans-cleavage are separated. Thus, a standard PCR amplification laboratory with independent areas of amplification and analysis rooms is required to prevent aerosol contamination while uncapping the amplification tubes and transferring the amplicons. To make the diagnosis more portable, Cas12a is then integrated with isothermal amplification methods such as RPA to create a one-pot detection system (Nguyen et al., 2020; Yue et al., 2021). The CRISPR reaction conditions have also been optimized, including the reaction buffer and the crRNA sequences, to further improve the cleavage efficiencies (Nguyen et al., 2020; Yue et al., 2021). In addition to fluorescent ssDNA reporters, different types of labeling and detection have been explored. For example, reporters can be dual labeled with FAM and biotin, and CRISPR-Dx can be combined with LFA technology (Bai et al., 2019; Broughton et al., 2020; Lu et al., 2020; Mukama et al., 2020) to facilitate the simple observation of diagnostic results on test strips without the requirement of ancillary instruments. Alternatively, the Cas12a-based trans-cleavage reaction can be integrated with cascade enzymatic and Fenton reactions, leading to a visible color change to indicate the diagnostic results (Huang et al., 2020a). Similar to the Cas9-based CRISPR-Chip technology that detects electrical outputs, the Cas12a-mediated trans-cleavage signals can also be read by electrochemical responses (Dai et al., 2019; Zhang et al., 2020) or solid-state nanopore sensors (Nouri et al., 2020), both of which can improve the detection sensitivities and reduce the detection time.

Cas12b is a type-Vb thermophilic Cas protein and exhibits trans-cleavage activities at higher temperatures (e.g., above 55°C). It can be combined with high-temperature isothermal reactions such as LAMP (loop-mediated isothermal amplification). With the use of *Alicyclobacillus acidoterrestris* Cas12b (AacCas12b) and LAMP, the first one-pot CRISPR-Dx system, namely, HOLMESv2, was developed, which markedly reduces the risk of aerosol contamination (Li et al., 2019a).

Cas12F, which is of type VF, also has trans-cleavage
Cas13-based CRISPR-Dx

The Cas13-family proteins instead recognize target single-stranded RNA (ssRNA) and trans-cleave collateral ssRNA sequences in the reaction system (Abudayyeh et al., 2016). With the combination of Cas13a and RPA isothermal amplification, the first CRISPR-Dx method using Cas13 trans-cleavage activities, namely, SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing), was developed, exhibiting atomic sensitivity and single-base mismatch specificity (Gootenberg et al., 2017). Because of the nonspecific properties of the trans-cleavage reactions, HOLMES and SHERLOCK are unable to detect multiple targets in one reaction, which limits the clinical applications of the CRISPR-Dx tools because an internal control is usually required in such scenarios. The multiplexed SHERLOCKv2 method was developed through the combination of Cas13 and Cas12a to solve this problem (Gootenberg et al., 2018). Different Cas13 orthologs have distinct cleavage preferences for dinucleotide RNA reporters, reflecting the orthogonality between Cas13 enzymes and enabling the detection of multiple targets in one pot. As Cas12 is orthogonal to Cas13, the multiplexity was further increased by the addition of Cas12a, achieving 4-channel multiplexing in a single SHERLOCKv2 reaction. In addition, SHERLOCKv2 successfully detects Dengue or Zika viruses and their mutants via LFA strips, which further highlights its potential as a multiplexable and portable nucleic acid detection system. Moreover, on the basis of SHERLOCK, dozens of methods have been recently developed and used in different scenarios, including COVID-19 diagnosis (Ackerman et al., 2020; Fozouni et al., 2021; Lee et al., 2020; Myhrvold et al., 2018; Patchsung et al., 2020; Shinoda et al., 2021; Zhou et al., 2021).

CRISPR-based non-nucleic acid detection

In addition to targeting nucleic acid diagnosis, CRISPR-Dx can also be used for the detection of non-nucleic acid (NNA) targets such as small molecules (Liang et al., 2019), metal ions and sodium ions (Xiong et al., 2020), proteins (Liu et al., 2021a), extracellular vesicles (EVs) (Zhao et al., 2020a; Zhao et al., 2020b) and cells (Li et al., 2021a). During NNA detection, NNA targets first lead to the generation of target nucleic acids that can be recognized by Cas proteins and trigger trans-cleavage reactions, which actually employ CRISPR-Dx as a signal amplifier. For example, in combination with allosteric transcription factors (aTFs), Cas12a can be used to rapidly detect small molecules from clinical samples (Liang et al., 2019). Specifically, target small molecules release aTFs from binding to dsDNA sequences, which serve as the target of Cas12a activator to trigger Cas12a-mediated trans-cleavage reactions.

In addition to aTFs, functional nucleic acid aptamers can be combined with Cas proteins to detect specific NNA targets, using both the high affinity and specificity of aptamers and high trans-cleavage activities of Cas proteins. Usually, the recognition and binding of NNA targets such as proteins or small molecules can be induced to change the conformation of aptamers, which then serve as Cas12a activators to produce trans-cleavage signals. In a recent study, allosteric aptamers were employed to recognize and bind the penicillin-binding protein 2a (PBP2a) protein on the surface of methicillin-resistant *Staphylococcus aureus* (MRSA), which resulted in the release of the initiator sequence to subsequently unfold a second hairpin probe to serve as the Cas12a activator. The introduction of Taq DNA polymerase and Nb.BbvCl nicking enzyme allows the continuous generation of Cas12a activators and enhances the detection sensitivity (Wei, 2021).

Similar strategies have been developed to detect target proteins on the surfaces of other bacteria, such as *Acinetobacter baumannii* (Li et al., 2020a) and tumor-derived EVs (Li et al., 2021b). After the binding of target proteins, aptamers can produce templates for *in vitro* transcription to generate crRNA and then initiate the Cas12a trans-cleavage reaction in a system that lacks crRNA (Zhao et al., 2020a). When aptamers are combined with immunoassays, one aptamer sequence is immobilized on the plate substrate to capture target proteins, and the other aptamer sequence then recognizes distinct structural conformations of the same target and links the Cas12a ternary complex to produce trans-cleavage signals (Li et al., 2021a). In addition, an aptamer-based CRISPR-Dx system can detect small organic molecules and metal ions, e.g., ATP and Na⁺ ions (Li et al., 2021a; Xiong et al., 2020), similar to protein detection. In combination with hybridization chain reaction (HCR), aptamers may bind to tumor EV proteins and initiate the generation of Cas12a activators by HCR, assisting in the direct detection of tumor EVs with high sensitivity (Xing et al., 2020).

Deoxyribozymes (DNAzymes) are another type of functional nucleotide that cleaves and releases specific DNA sequences in the presence of certain metal ions. Using this strategy, DNAzymes are integrated with the Cas12a trans-cleavage system to facilitate the convenient and sensitive detection of ions such as Pb²⁺ (Li et al., 2020a) and Na⁺ (Xiong et al., 2020).
**CRISPR-Dx in the COVID-19 pandemic**

The outbreak of the COVID-19 pandemic has caused millions of deaths worldwide and is still disrupting livelihoods and threatening human life and health. Diagnostic tools for the pathogenic SARS-CoV-2 virus that use either immunochromatography or reverse transcription (RT) real-time PCR (RT-qPCR) technology were successfully developed soon after the outbreak (Corman et al., 2020; He et al., 2020). Although the immunology solution is of great convenience, the low accuracy and sensitivity are a concern. PCR, in contrast, is well known for its robustness and accuracy; however, the need for specialized machines and laboratories may prevent it from being used in decentralized scenarios. With the aid of CRISPR technologies, dozens of CRISPR-Dx systems have been developed, aiming to provide SARS-CoV-2 diagnosis with high specificity, sensitivity, rapidness, and convenience (Ding et al., 2020; Fozouni et al., 2021; Ganbaatar and Liu, 2021; He et al., 2020; Huang et al., 2020b; Joung et al., 2020; Ning et al., 2021; Patchsun et al., 2020; Shinoda et al., 2021).

By integrating Cas trans-cleavage reactions with either PCR or isothermal amplification, the CRISPR-Dx methods can further improve the specificity and sensitivity of amplification methods (Huang et al., 2020b; Joung et al., 2020). For example, due to the limitations of RT-qPCR technology, high Ct-values are frequently encountered during SARS-CoV-2 diagnosis, possibly resulting in both false-positive and false-negative diagnostic results (Li et al., 2020c; Wernike et al., 2021; Xiao et al., 2020). HOLMES was employed to solve this problem, and the RT-qPCR amplicons can be further analyzed by Cas12a-based diagnosis, establishing a specific enhancer for the identification of nucleic acids amplified by a PCR (SENA) diagnostic system with improved sensitivity and specificity (Huang et al., 2020b). Similarly, Cas12a has been combined with RT-RPA to develop a sensitive diagnostic system for SARS-CoV-2 detection (Ning et al., 2021).

LAMP has been demonstrated as an efficient isothermal method for nucleic acid amplification and detection, but it has a high false-positive rate. To solve this problem and achieve convenient SARS-CoV-2 detection, researchers developed the one-pot STOPCovid.v2 method, which resembles HOLMESv2 in principle (Li et al., 2019a), with the use of LAMP for preamplification and thermophilic *Alcyclobacillus acidophilus* Cas12b (AapCas12b) for trans-cleavage and signal output (Joung et al., 2020). Because AapCas12b is resistant to high temperature, STOPCovid.v2 can be carried out at 60°C to facilitate LAMP pre-amplification and exhibits a higher detection sensitivity than HOLMESv2, which is performed at 55°C (Li et al., 2019a). In addition, magnetic beads are used to enrich the total nucleic acids from the lysed samples, which further enhances the detection sensitivity of STOPCovid.v2 (i.e., 33 copies per milliliter) far beyond the requirement of the Centers for Disease Control and Prevention (CDC) for the RT-qPCR test (Joung et al., 2020). As no more expensive instrument than a heat block is required, STOPCovid v2 can be used to facilitate COVID-19 diagnosis in areas lacking sophisticated medical instruments.

Several amplification-free CRISPR-Dx systems using Cas trans-cleavage activities have been successfully developed and shown competitiveness in COVID-19 diagnosis (Fozouni et al., 2021; Liu et al., 2021a; Shi et al., 2021; Shinoda et al., 2021; Tian et al., 2021; Yue et al., 2021). To increase the detection sensitivity, more than one crRNA targeting the SARS-CoV-2 RNA sequence can be used, and amplification-free detection can be accomplished within 30 min with a sensitivity of approximately 100 copies per microliter (Fozouni et al., 2021); however, this sensitivity may still require improvement to meet the CDC criteria. Alternatively, unamplified target nucleic acids can be remarkably concentrated in picolitre-sized droplets with the ultralocalization of Cas13 trans-cleavage reactions by means of droplet microfluidics (Tian et al., 2021), and the system shows extremely high sensitivity and enables the absolute digital quantification of target RNAs at the single-molecule level without preamplification.

**Summary**

CRISPR-Dx began in 2015 when the CASFISH system with CRISPR-dCas9 was developed (Deng et al., 2015). The discovery of the trans-cleavage activities of Cas12 and Cas13 has aroused further interest in developing next-generation molecular diagnostic tools (Chertow, 2018). Because of the outstanding performance, two CRISPR-Dx products were issued an Emergency Use Authorization (EUA) for COVID-19 diagnosis last year, including the Cas13-based Sherlock CRISPR SARS-CoV-2 Kit by the Food and Drug Administration (FDA) in the US and the Cas12-based 2019-nCoV Nucleic Acid Detection Kit (CRISPR Immunology) by the National Medical Products Administration (NMPA) in China. With an increasing number of in vitro diagnostic (IVD) products on the market, CRISPR-Dx might eventually enter clinical use.

Because of the specificity and the trans-cleavage activity of Cas proteins, CRISPR-Dx systems have shown great advantages in detection, including sensitivity, accuracy, rapidness, and portability (Li et al., 2019b). Several amplification-free CRISPR-Dx systems have been developed using either Cas12 or Cas13 (Fozouni et al., 2021; Liu et al., 2021a; Shinoda et al., 2021; Tian et al., 2021) with optimization of the reaction system and readout-related sensitivity. They show great potential in the diagnosis of pathogens and tumors. Although Cas9 can be integrated with a graphene-based field-effect transistor to achieve the amplification-free
Since 2015, more than 10 gene therapy products have been approved for use in neurodegenerative disorders, infectious diseases, cancer, etc. The total number of clinical studies in this area has surpassed 2,500, targeting monogenic diseases, complex neurodegenerative disorders, infectious diseases, cancer, etc. The limited duration of AAV vector and random insertion-induced lentiviral vector tumorigenic potential are major disadvantages compared to the genetic modification of patient cells. However, the genetic modification of patient cells is potentially dangerous, so safety issues are the most critical factors that have raised serious concerns in gene therapy clinical trials. The data accumulated since the 1990s make it clear that the risks fall into two main aspects. The risk of insertional mutagenesis is a recognized disadvantage of integrating vectors. The clinical ramifications of insertional mutagenesis were confirmed by the development of T cell leukemia in four young patients who received retrovirus-mediated gene therapy for X-linked severe combined immunodeficiency (SCID) (Haack-Bey-Abina et al., 2008). Second, immune-mediated rejection is related to in vivo vector vehicles (Shirley et al., 2020). For example, adenoviral vectors (AdVs) may induce a strong immune response that leads to life-threatening multiple organ system failure (Raper et al., 2003). Currently, vectors that cause severe immune side effects have been weeded out. Lentiviral vectors have become the leading system for ex vivo gene transfer (Naldini, 2011), while in vivo therapies usually use AAV vectors (Wang et al., 2019a; Witzigmann et al., 2020). AAV is considered a relatively safe vector since it induces minimal immune responses. Its vector DNA predominantly forms a stable episome that prevents insertional mutagenesis at the cost of expression duration. The limited duration of AAV vector and random insertion-induced lentiviral vector tumorigenic potential are major deficiencies of “traditional” gene therapy.

In contrast to traditional gene therapy technologies, which can mediate only “gene addition” through various delivery vectors, genome editing technologies, such as ZFNs, mega-nucleases, TALENs and Cas9 nucleases, are able to generate permanent, precise and flexible gene ablation, insertion or correction at one or multiple target genes. The versatility and simplicity of the revolutionary programmable nucleases, especially the CRISPR/Cas9 system, quickly made them the most critical technology in gene therapy. This chapter provides a brief summary of recent progress in genome editing-mediated gene therapy, from bench to bedside.

Therapeutic gene editing

The goal of gene therapy is to use various strategies to correct or manipulate gene expression to restore defective cell functions or reinforce the biological properties of normal cells to ameliorate symptoms or cure diseases with minimal adverse events. Since the first clinical study undertaken at the US National Institutes of Health in 1990, more than three decades of exploration have built a solid basis for gene therapy. The total number of clinical studies in this area has surpassed 2,500, targeting monogenic diseases, complex neurodegenerative disorders, infectious diseases, cancer, etc. Since 2015, more than 10 gene therapy products have been approved worldwide for cancer and genetic disorders. Like the commentary published in Science, we would like to say “gene therapy comes of age” (Dunbar et al., 2018).

There are two basic delivery strategies to fulfill the goal of gene therapy: (i) introducing an integrating vector with genetic material into proliferating cells (usually hematopoietic cells) through an ex vivo process so that the donated DNA will be replicated during cell division and therefore passed to every daughter cell; or (ii) introducing exogenous nucleic acids into postmitotic cells via nonintegrating vectors through in vivo methods to achieve sustained expression of the target gene in the entire lifespan of the cells. However, the genetic modification of patient cells is potentially dangerous, so safety issues are the most critical factors that have raised serious concerns in gene therapy clinical trials. The data accumulated since the 1990s make it clear that the risks fall into two main aspects. The risk of insertional mutagenesis is a recognized disadvantage of integrating vectors. The clinical ramifications of insertional mutagenesis were confirmed by the development of T cell leukemia in four young patients who received retrovirus-mediated gene therapy for X-linked severe combined immunodeficiency (SCID) (Haack-Bey-Abina et al., 2008). Second, immune-mediated rejection is related to in vivo vector vehicles (Shirley et al., 2020). For example, adenoviral vectors (AdVs) may induce a strong immune response that leads to life-threatening multiple organ system failure (Raper et al., 2003). Currently, vectors that cause severe immune side effects have been weeded out. Lentiviral vectors have become the leading system for ex vivo gene transfer (Naldini, 2011), while in vivo therapies usually use AAV vectors (Wang et al., 2019a; Witzigmann et al., 2020). AAV is considered a relatively safe vector since it induces minimal immune responses. Its vector DNA predominantly forms a stable episome that prevents insertional mutagenesis at the cost of expression duration. The limited duration of AAV vector and random insertion-induced lentiviral vector tumorigenic potential are major deficiencies of “traditional” gene therapy.

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In vivo gene therapy

Genetic diseases of the nervous system, vision, and hearing loss as examples

Combining the progress of several research areas, including
molecular biology, human genetics, delivery approaches, and clinical translation, retinal gene therapy is progressively being recognized as a potential intervention with great potential for treating inherited retinal dystrophies (IRDs). Early proof-of-concept clinical trials (NCT00481546, NCT00516477, and NCT00643747) used AAV vectors to deliver the gene encoding a 65-kD retinal pigment epithelium-associated protein (RPE65) into the eyes of patients carrying biallelic RPE65 recessive mutations (Bainbridge et al., 2008; Cideciyan et al., 2009; Maguire et al., 2008). Subsequent studies and clinical trials (NCT00481546, NCT00643747, NCT01208389, and NCT00999609) demonstrated a statistically significant and clinically meaningful difference between the intervention and control groups without significant safety issues (Bainbridge et al., 2015; Bennett et al., 2012; Bennett et al., 2016; Jacobson et al., 2015; Maguire et al., 2009; Russell et al., 2017). All these efforts allowed the FDA and EMA to approve Luxturna (Spark Therapeutics) at the end of 2017 as a gene therapy product to treat the monogenic disorder Leber congenital amaurosis (LCA-2). The approval of Luxturna fueled additional trials for other IRDs (Chiu et al., 2021) focused on LCA (NCT00481546, NCT02946879), retinitis pigmentosa (RP) (NCT03328130, NCT04611503), X-linked retinitis pigmentosa (XLRP) (NCT04671433, NCT03316560), and achromatopsia (NCT02341807, NCT02077361). In addition to AAV-mediated gene therapy, a few studies have tried to correct the mutation at the mRNA level via antisense oligonucleotides to restore the splicing of mutant pre-mRNA (NCT03913143) (Chiu et al., 2021).

Most recently, preclinical studies have demonstrated the promising application of CRISPR-Cas9-based therapeutics in LCA, RP, or other retinal diseases. After Zhong et al. (2015) used the CRISPR-Cas9 system to validate that the expression of KCNJ13 is related to photoreceptor survival and plays a role in LCA pathogenesis, researchers employed it to edit a disease-associated gene in LCA. IVS26, an adenine-to-guanine point mutation (c.2991+1655A>G) in intron 26, is a common mutation involved in LCA subtype 10 (LCA10) (Boyce et al., 2014; den Hollander et al., 2006). This mutation forms a new splicing donor site in the mRNA, creating a premature stop codon that completely inactivates CEP290. Thus, the Cas9 proteins from S. pyogenes or S. aureus with dual sgRNAs were tested to remove or reverse the mutation in the CEP290 gene to correct splicing and then generate a functional CEP290 transcript. The experimental data suggested that Cas9-based removal of the pathogenic variant in the intron of CEP290 could be a potential treatment strategy for LCA (Maeder et al., 2019; Ruan et al., 2017). In the first Cas9-mediated in vivo gene therapy trial, Allergan and Editas Medicine initiated the first patient dosing in the phase 1/2 clinical trial of AGN-151587 (EDIT-101) for the treatment of LCA10 (NCT03872479) with the above strategy. In this trial, SaCas9 and sgRNAs were packaged in AAV vectors and delivered via subretinal injection.

As a monogenic hereditary retinal disorder, RP causes irreversible blindness due to pathogenic (loss-of-function or gain-of-function) mutations in more than 50 candidate genes. Therefore, the CRISPR-Cas9 genome editing system is a prominent tool to treat autosomal dominant RP (adRP). For example, in rodent models, a recent study employed CRISPR-Cas9 to knock out the mutant allele of the rhodopsin (RHO) gene, which is vital for retinal cell survival, in rodent models (Bakondi et al., 2016). The disruption of the murine S334ter mutation (Rho(S334)) halted retinal degeneration and improved visual function. In three other similar human studies, the modification P23H in the RHO gene by CRISPR-Cas9 significantly reduced the abundance of mutant RHO protein (Giannelli et al., 2018; Latella et al., 2016; Li et al., 2018a). Moreover, this genome editing system has been utilized to understand the molecular biology of RP for clinical interventions. For example, a nonsense point mutation (Y347X) and an Xmv-28 insertion in the phosphodiesterase 6B (PDE6B) gene are associated with RP in a rodless (rd1) mouse model. One study demonstrated that the outer nuclear layer (ONL) could be restored after Cas9 repair of the Y347X mutation (Wu et al., 2016). In another study, Cas9 was assembled with the E. coli RecA protein to repair this mutation in rd1 mice. The most important contribution of this study is the demonstration of the feasibility of Cas9-induced HDR to treat rd1 disease and of the Cas9/RecA system to improve HDR efficiency in vivo, which may also benefit the treatment of other diseases (Cai et al., 2019).

A large number of people suffering from hearing loss would also benefit from gene-editing technologies, since half of hearing loss is caused by genetic mutations, among which 20% are gain-of-function mutation (Müller and Barr-Gillespie, 2015). Theoretically, these mutant alleles can be targeted by CRISPR-Cas9 to correct the production of malfunctioning proteins. TMC1 is an essential protein for the conversion of mechanical signals to bioelectrical signals (Pan et al., 2013). The TMC1 c.T1253A mutation can cause the hearing loss disorder DFNA36, a dominant genetic disorder. To test whether disruption of the c.T1253A allele via Cas9 is able to rescue hearing loss in a DFNA36 mouse model. Gao et al. (2018) utilized the lipid delivery system to transmit Cas9/sgRNA into the inner ear of a mouse model. They designed a series of sgRNAs containing the TMC1 mutation at the PAM proximal region to prevent cleavage of the WT TMC1 allele. Their results showed that the treatment robustly reduced progressive hearing loss, with higher hair cell survival rates and lower auditory brainstem response thresholds in the neonatal mouse model. In contrast to Gao’s strategy, György et al. (2019) used AAV2/Anc80 as the delivery vehicle and SaCas9-KKH as the targeting nuclease.
They placed the TMC1 mutation in the PAM region to selectively target the mutant TMC1 allele and found that the postnatal delivery of AAV-SaCas9-KKH/sgRNA durably preserved the hearing capability of the mice for at least one year. However, some TMC1 mutations, for example, c.A545G, result in a recessive loss-of-function point mutation, which is not suitable for the above disruption strategy. To correct the point mutation and rescue the hearing ability of TMC1 c.A545G mutated mice, Yeh et al. (2020) tested several base editors. They chose the most efficient AID-BE4max as the effector and selected the split-intein delivery system to fit the base editor into a dual AAV package system. They achieved approximately 2.3% desired base correction in Tmc1 with ear injection of the dual AAV. Importantly, mice with this correction rate could produce normal Tmc1 mRNA that reached 51% of the WT level, which restored the low-frequency hearing of the mice 4 weeks after the treatment.

Both retinal and hair cells are suitable targets for genome editing-based gene therapy, since they can be efficiently transduced through local injection via AAV, and the dose of AAV required is much lower than the systemic injection doses required for other diseases. Notably, even limited editing efficiency demonstrated impressive efficacy in these studies. This suggests that editing efficiency may not be highly demanded in these applications.

**Gene editing in liver gene therapy**

The liver is one of the most targeted organs for gene therapy because many metabolic diseases are caused by the dysfunction of genes related to the liver. In addition, the liver can be efficiently transduced by viral or nonviral vectors. In early clinical trials, researchers utilized the gene addition technique to deliver healthy genes into the liver via lentivirus or AAV. Several clinical trials aiming to cure hemophilia had already shown encouraging results before the new gene-editing tools were developed (Nguyen et al., 2021). The development of efficient delivery technologies and the advances in gene editing tools have resulted in practical approaches to treat several genetic diseases via NHEJ or HDR in the liver, including hereditary tyrosinemia-I (HT-I) and primary hyperoxaluria type I (PH-I), targeting an upstream gene to reprogram the metabolic pathway could also be an effective approach. In HT-I, mutations in the gene fumarylacetoacetase (FAH) result in the accumulation of tyrosine and other toxic metabolic intermediates, such as succinylacetone (Shao et al., 2014). The current treatment for HT-I requires the daily ingestion of nitisinone, an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPD), which is the upstream enzyme for tyrosine degradation (Holme and Lindstedt, 1998). The inhibition of HPD will convert the accumulation of toxic succinylacetone into its upstream intermediate—4-hydroxyphenylpyruvate (4HPP), which is observed in the more benign disease HT-III (Pankowicz et al., 2016). Following the above mechanism, Pankowicz et al. (2016) delivered Cas9/sgRNA targeting the Hpd1 gene into the murine liver via the hydrodynamic tail vein injection of plasmids. A week after the injection, up to 20% of the Hpd allele was deleted. As the edited hepatocytes have a growth advantage over diseased cells, they quickly repopulated 99% of hepatocytes 8 weeks after treatment.

Similar to the treatment of HT-I, metabolic reprogramming was performed in the treatment of PH-I. In PH-I, a mutation in the AGXT gene leads to the failure of glyoxylate to glycine conversion. The accumulated glycosylate is oxidized by lactate dehydrogenase (LDH), causing the overproduction of highly insoluble calcium oxalate (CaOx) crystals in the kidneys (Zheng et al., 2020). An ongoing clinical trial to treat
PH-I targeting the HAO1 gene via RNA interference reduces the expression of glycolate oxidase, an upstream enzyme that generates glyoxy late (Garrelfs et al., 2021) (NCT03681184). Soon, the therapeutic effect of HAO1 disruption through CRISPR/Cas9 was also validated in mice (Zabaleta et al., 2018) and rats (Zheng et al., 2020). In both studies, the treated animals exhibited alleviated symptoms, showing that this strategy has great promise. Notably, although the use of CRISPR/Cas9 or other nucleases to treat genetic diseases via deletion of endogenous genes to reprogram metabolic pathways is effective, the long-term safety issue of this strategy has yet to be extensively examined. Due to these concerns, current clinical trials have focused mainly on RNAi strategies to treat those diseases through in vivo gene manipulation. However, an advantage of programmable nucleases is that they avoid the repeat delivery of therapeutic vectors, which could be painful and costly to patients. Recently, Intellia and Regeneron revealed milestone clinical data on therapy for transthyretin (ATTR) amyloidosis, a disease caused by misfolding and deposition of the transthyretin protein. Their study delivered nanoparticles encapsulating the mRNA of Cas9 and sgRNA targeting the transthyretin (TTR) gene into the patients. Taking advantage of mRNA avoids the long-term in vivo expression of Cas9, thus minimizing the possibilities of off-target events and exogenous DNA integration. More importantly, they demonstrated that a single dose of NTLA-200 was able to reduce transthyretin by 87% on average, which lasted for over a year without relapse (NCT04601051) (Gillmore et al., 2021). As the capacity of AAVs is limited (approximately 4.7 kb), it is difficult to deliver genome-editing materials through a single AAV vector. The clinical success of LNP-based Cas9/sgRNA delivery is a critical landmark for both mRNA medicine and genome editing therapeutics, as the short life of mRNA would greatly reduce the potential off-targeting effects, and the LNPs would not induce immune tolerance, which is the problem with repetitive dosing of AAV products in patients with AAV antibodies.

In vivo gene therapy via DNA fragment integration may be the greatest challenge in this field because conventional DNA integration use the HDR pathway, which has an extremely low intrinsic frequency, especially in quiescent somatic cells. However, in some diseases, partial correction of the mutated genes might significantly and durably relieve their symptoms. A case in point is the potential gene therapy for hemophilia. Guan et al. (2016) achieved a 0.5% correction of the f9 gene through hydrodynamic tail vein injection of the Cas9/sgRNA plasmid and a donor fragment into the mouse liver. They found that the treated mice had shortened blood coagulation time and survived the tail-clip challenge, although only a tiny portion of the liver cells was corrected. Similar results were observed in the study for hemophilia A (Chen et al., 2019a). To further increase the integration frequency of large DNA fragments, Zhang et al. (2019a) harnessed NHEJ-mediated knockin and an AAV donor fragment to treat hemophilia A. They achieved an in vivo knockin frequency of 2% for the BDFFP donor fragment (over 4.4 kb). Since AAV vectors are commonly used as delivery vectors for nucleases and donors in gene therapies, an increase in delivery efficiency might elevate both cleavage and HDR efficiency. Yin et al. (2020) attempted to code deliver an AAV receptor (AAVR) in a study on gene therapy for phenylketonuria (PKU). The coexpression of AAVR significantly increased the in vivo HDR efficiency by up to 20-fold and ameliorated the blood Phe level, although the long-term effects of AAVR overexpression need full investigation in the future.

It is worth mentioning that most of the above editing strategies are based on the creation of DSBs in the genome. However, due to the potential risks of DSBs, researchers are making gene-editing tools more precise on the one hand, while on the other hand, they are looking for potential substitutes for the programmable nucleases. The invention of base editors offered scientists alternative choices. Unlike conventional programmable nucleases, base editors produce few DSBs in the genome, and their efficiency outperforms the traditional HDR pathway. Villiger et al. (2018) attempted to treat a PKU mouse model via in vivo base editing. They engineered the cytidine base editor (ABE) into an intein-split system to fit each portion of the base editor into the capacity limitation of the AAV particles. The AAV was then delivered via tail vein injection. During the next 26 weeks, they observed a continuous increase in the DNA and mRNA correction rates and a significant drop in the L-Phe level in the treated mice, indicating the feasibility of this strategy. Moreover, the high efficiency and flexibility of base editors give researchers a greater capability to cure diseases. Beyond correcting mutated base pairs, scientists are able to manipulate mRNA splicing, including creating de novo start codons and installing stop codons in the genome. In 2020, the first gene therapy study evaluating ABE was performed by Song et al. They combined ABE and the hydrodynamic tail vein injection technique to restore a mutated splice donor site in the fah gene in an HT-I mouse model (Song et al., 2020). In the same year, Yang et al. (2020) managed to create a de novo “ATG” via in vivo base editing, which helped to read through the mutant fah gene in an HT-I mouse model. Recently, Rothgangl et al. (2021) used an ABE to target the splice donor site in intron 1 of the PCSK9 gene in macaques. Their strategy led to a decrease of 95% in the PCSK9 level within 48 h, and more importantly, a corresponding decrease of 58% in LDL was observed as well.

While base editors are efficient and effective in gene therapy studies, two major concerns affect the research field. First, the bystander editing effect could narrow the potential target scope of the base editors. Second, with the emergence
of more sensitive detection methods, new off-target phenomena of base editors could be discovered (Lei et al., 2021). Therefore, a systematic off-target event inspection is suggested for each target site before a clinical trial is initiated.

**Ex vivo gene therapy**

**Gene therapy in hematopoietic stem cells**

Due to the maturation of blood separation techniques, *ex vivo* gene therapies mainly adopt hematopoietic progenitor/stem cells (HSPCs) as the target. Initially, clinical trials took advantage of gene addition technology to treat inherited immunodeficiency disorders, including X-linked SCID and adenosine deaminase (ADA) deficiency. In the above trials, healthy donor genes encoding the γc or ADA protein were delivered into the immune cells or later into the HSPCs of the patients via retroviral or lentiviral particles. Treated cells were then transplanted back to the patients to supplement the proteins needed as the building blocks of the immune system. The overall efficacy of the first attempts was quite exciting. Although the therapeutic effect lasted only months in some cases, others demonstrated that the concept of long-term gene augmentation was feasible (Aiuti et al., 2002; Aiuti et al., 2009).

Based on prior knowledge, when it became plausible that genetic disorders could be cured by HSPC manipulation and transplantation, β-thalassemia and sickle cell disease were the first diseases to be targeted. Both diseases are caused by mutations in the gene encoding β-globin, which normally forms the hemoglobin tetramer with α-globin. Patients with a severe type of both diseases have low oxygen-carrying hemoglobin and depend on regular blood transfusions (Thompson et al., 2018). Reactivation of the silenced gene γ-globin, which acts as a substitute for β-globin, could cure these two diseases. More importantly, studies suggested that γ-globin could be reactivated by creating indels in the regulatory elements of the γ-globin promoter (−112 to −117) region (Traxler et al., 2016) or of the +58 enhancer of a γ-globin repressor, namely, BCL11A (Canver et al., 2015; Wu et al., 2019). Immediately, various gene-editing studies were performed using HSPCs to prove this concept via ZFN (Bjurström et al., 2016), TALEN (Humbert and Kiem, 2015), or, in most cases, via CRISPR/Cas9 (Canver et al., 2015; Wu et al., 2019). In addition, edited HSPCs from patients were viable and functional in nonhuman primates (Humbert and Kiem, 2015; Humbert et al., 2018). In 2018, gene editing companies, including Sangamo and CRISPR Therapeutics, initiated phase 1/2 clinical trials evaluating the efficacy and safety of editing the BCL11A enhancer to treat sickle cell disease (NCT03745287) or β-thalassemia (NCT03432364, NCT03655678). A year later, similar trials targeting BCL11A were launched in China (NCT04211480, NCT04390971). The results from these trials are astounding. All patients receiving the treatment are able to produce sufficient fetal hemoglobin, which helps them become independent of transfusions (Frangou et al., 2021). In addition to the γ-globin reactivation strategy for β-thalassemia, scientists discovered other methods to restore β-globin. For example, in β-thalassemia IVS-654, a C654-A transition leads to aberrant β-globin pre-mRNA splicing, preventing the synthesis of β-globin protein. Using programmable nucleases, scientists successfully restored β-globin mRNA splicing by targeting intron 2 of the β-globin gene (Xu et al., 2015). Another more direct strategy for β-globinopathies is to correct the disease-causing mutations via CRISPR/Cas9-stimulated HDR (Dever et al., 2016; Wilkinson et al., 2021). However, since HDR has a low spontaneous frequency, whether enough corrected HSPCs can be enriched is the key to the success of the treatment. Currently, a clinical trial based on the HDR strategy has been initiated (NCT04819841) by Graphite Bio. Notably, in addition to nuclease-based gene-editing tools, base editors are beginning to play an important role in the treatment of β-globinopathies. Reports have revealed that it is possible to reactivate γ-globin by editing the BCL11A +58 enhancer (Zeng et al., 2020) or HBG1/2 promoter region (Wang et al., 2020b). Recently, results obtained by Gregory et al. demonstrated that through an adenine base editor (ABE8e-NRCH), the GAG(E) to GTG(V) mutation, which causes sickle cell disease, could be efficiently converted to a benign Makassar variant (Newby et al., 2021). Since ABES are able to convert base pairs efficiently without inducing DSBs, which is superior to Cas9-mediated mutagenesis, the sgRNA-independent off-targeting effects of the base editors still require close attention. Scientists are devoting attention to improving base editing technology through engineering base editors and developing delivery technologies for Cas9 protein or mRNA to reduce off-target edits.

**Gene editing in primary T cells for immunotherapy**

In 1994, zinc finger proteins were first shown to repress the function of oncogenic BCR-ABL fusion sequences by directly binding DNA (Choo et al., 1994). Later, zinc finger proteins were coupled with nuclease to mediate gene disruption in various cell lines and primary cells, such as T cells (Urnov et al., 2005). A double genetic disruption of TCR-β and α-chain genes followed by the lentiviral transfer of a new TCR into human primary T cells was first reported in 2012 (Provasi et al., 2012). The edited T cells treated with ZFNs lacked surface expression of CD3-TCR and expanded with the addition of interleukin-7 (IL-7) and IL-15. Another study also disrupted TRAC, TRBC1, and TRBC2 using ZFNs to generate universal chimeric antigen receptor (CAR) T cells for multiple allogenic patients (Torikai et al., 2012). Later, the same group used ZFNs to knock out the HLA-A gene in CAR-T cells, further improving the universal CAR-T
strategy (Torikai et al., 2013). Another study knocked down the glucocorticoid receptor using ZFNs in modified cytolytic T lymphocytes (CTLs), which were generated by introducing a chimeric T-cell receptor consisting of an extracellular IL-13 domain and a cytoplasmic CD3 domain (IL-13-zetakine). Thus, ZFN-modified glucocorticoid-resistant IL-13-zetakine-targeted CTLs retained their function in cancer patients regardless of glucocorticoid treatment (Reik et al., 2008).

Only one year after the elucidation of TALE-DNA recognition codes, TALEs were designed and linked to FokI to establish the TALEN platform. It is worth mentioning that after scaffold optimization, TALE showed comparable on-target activity to benchmark ZFNs but lower cytotoxicity, at least at three human loci tested (CCR5, IL2RG, AAVS1) (Mussolino et al., 2011; Mussolino et al., 2014). The first TALEN-mediated cell therapy to enter clinical trials has focused mainly on the generation of universal CAR-T cells. Cellectis SA has developed an allogeneic approach named universal chimeric antigen receptor (CAR) T-cells targeting CD19 (UCART19), which aims to offer standardized therapies with good consistency and immediate availability. Genetic manipulation involves TALEN mRNA-mediated gene disruption against TCRα constant gene (TRAC) and CD52. The preliminary results reported two phase I clinical trials to evaluate the safety and antileukemic activity of UCART19 in children and adults with relapsed or refractory B-cell acute lymphoblastic leukemia (Benjamin et al., 2020). Fourteen (67%) of 21 patients had a complete response or complete response with incomplete hematological recovery 28 d after infusion. Patients not receiving CD52 targeting alemtuzumab (n=4) showed no UCART19 expansion or antileukemic activity. The median duration of response was 4.1 months, with ten (71%) of 14 responders proceeding to a subsequent allogeneic stem-cell transplant. Progression-free survival at 6 months was 27%, and overall survival was 55% (NCT02808442, NCT02746952). A similar strategy was applied to other CAR-T products in the Cellectis pipeline, such as UCART123 targeting CD123, the primary low-affinity subunit of the IL-3 receptor, which is highly expressed in some hematological cancers. The phase I UCART19 trial was underway for AML (NCT03190278). Several other TALEN-edited allogeneic UCART products have also entered phase 1 clinical trials, including UCART22, UCARTCS1, and ALLO-751 (NCT040993596, NCT04142619, NCT04150497).

The first CRISPR/Cas9-mediated multiplex gene editing in CAR-T cells was reported by the June group and Wang group (Liu et al., 2017b; Ren et al., 2017). TRAC, B2M, and PDCD1 were chosen as target genes in both studies. Electroporation of Cas9 mRNA-sgRNAs was applied in June’s study. After optimizing the electroporation conditions for delivery, 80% single-gene KO efficiency and 65% dKO efficiency were obtained. Instead of Cas9 mRNA, the Wang group delivered a RNP consisting of the Cas9 protein in complex with sgRNAs and achieved efficient single- and multiple-gene KO. In both studies, potent antitumor efficacy of gene-edited CAR-T cells was demonstrated in both in vitro assays and tumor xenograft mouse models. Later, CRISPR/Cas9-mediated KO of various genes in CAR-T cells or CD8+ T cells to eliminate various immune checkpoint genes (PDCD1, LAG3, CTLA4, DGKα) (Jung et al., 2018; Rupp et al., 2017; Zhang et al., 2019b; Zhang et al., 2017b), to inhibit immune-suppressive signaling (TGFβ, A2AR) (Li et al., 2020b), or to generate allogeneic CAR-T cells for the treatment of T cell hematologic malignancies (CD7&TRAC) was reported (Cooper et al., 2018). It is worth noting that triphosphate at the 5’ end of IVT (in vitro transcription)-sgRNA could initiate the innate immune responses of primary T cells, impairing cell viability. Eliminating the triphosphate at the 5’ end of IVT-sgRNA by CIP or using chemically synthesized sgRNA solved this problem (Kim et al., 2018; Mu et al., 2019). As with TALENs, the first CRISPR trial for gene-edited cells was also T cells. They were first applied to treat a patient with advanced non-small cell lung cancer (NCT02793865). Many subsequent registered CRISPR trials focused on the use of autologous T-cells, knocking out the immune checkpoint inhibitor programmed cell death-1 (PD1) prior to reinfusion. Seven clinical trials targeting PD1 are currently in phase 1 (NCT02793856, NCT02863913, NCT02867332, NCT02867345, NCT03545815, NCT03747965, NCT04417764), mainly in China.

**Gene editing in infectious diseases**

The main strategy of antiviral therapeutics involves altering the host genes required for viral infection or targeting the viral genes essential for viral productivity (Kennedy and Cullen, 2017). The strategy of genome editing-based HIV therapy is to edit the gene encoding the receptor of HIV infection in CD4+ T or CD34+ hematopoietic stem/progenitor cells and reinfuse the modified cells into patients.

The CCR5 gene encodes the HIV coreceptor, which is utilized by HIV-dominant strains. Homozygosity of the naturally occurring 32 bp deletion (delta32) in CCR5 confers resistance to HIV-1 infection (Liu et al., 1996), and people heterozygous for delta32 have a slower progression after HIV infection, suggesting the feasibility of obtaining HIV resistance by mutating CCR5 using genome editing technologies (Huang et al., 1996; Zimmerman et al., 1997). The pioneering work in this field was done using ZFNs. In a preclinical study, Perez et al. delivered ZFN into human primary CD4+ T cells using a chimeric Ad5/F35 adenoviral vector and achieved a 50% CCR5 disruption rate. HIV-1-infected mice engrafted with CCR5-edited T cells had lower viral loads and higher CD4+ T-cell counts than mice engrafted with control T cells (Perez et al., 2008). Based on this promising therapeutic outcome, the first clinical usage of
gene-edited cell therapy was conducted in 2009 by the University of Pennsylvania in collaboration with Sangamo Therapeutics, aiming to disrupt CCR5 by using ZFNs in autologous CD4+ T-cells of HIV patients (NCT00842634) (Tebas et al., 2014). The clinical results were revealed in 2014 with only 1 serious adverse event due to a transfusion reaction, proving that the method was safe. After treatment, blood HIV DNA was decreased in all patients, and HIV RNA became undetectable in one patient, suggesting the feasibility of clinical application of gene editing. In addition to CD4+ T cells, CD34+ HSPCs were also engineered to provide a more durable source of HIV-resistant T cells (Allen et al., 2018). Clinical trials using ZNFs (NCT02500849) and CRISPR/Cas9 (NCT03164135) to disrupt CCR5 in CD34+ HSPCs have also been reported (Xu et al., 2019). Although the successful transplantation and long-term engraftment of CRISPR-edited HSPCs were observed, the percentage of CCR5 disruption in lymphocytes was only approximately 5%, which indicates the need for further improvement of this approach (NCT03164135). In addition, TALEN was used to disrupt CCR5, with relatively lower cytotoxicity and higher specificity than ZFNs (Mussolino et al., 2014).

In addition to CCR5, C-X-C chemokine receptor 4 (CXCR4) is an important coreceptor for CXCR4-tropic HIV-1. Thus, HIV-resistant CD4+ T cells that simultaneously inactivate the coreceptors CCR5 and CXCR4 confer protection against HIV that uses either of the coreceptors (Digidu et al., 2014). Similar ex vivo work was also performed using the CRISPR/Cas9 platform (Hendel et al., 2015; Mandal et al., 2014). Instead of direct disruption of the viral genome, researchers have also created alternative methods to eliminate HIV infection. For example, taking advantage of CRISPRa, a gene activation system that consists of a catalytically dead Cas9 and a transcriptional activation domain, scientists are able to reactivate HIV from its latency state (Ji et al., 2016; Saayman et al., 2016). As a result of latency reversal, cells that carry dormant HIV are exposed to immune cells and eliminated.

Infection with high-risk human papillomavirus (HR-HPV) is the main cause of cervical cancer, usually due to the persistent expression of HR-HPV oncogenes E6 and E7. Using gene editing to disrupt viral oncogenes is a potential therapeutic strategy. The proof-of-concept work was done by the Wang group in 2014 using ZFNs against E7 DNA in HPV16/18-positive cervical cancer cells. Repressed xenograft formation was also evidenced in vivo (Ding et al., 2014b). Later, they reported similar work using the CRISPR/Cas system (Hu et al., 2014) and TALENs (Hu et al., 2015). Based on these promising preclinical data, four clinical trials were registered to study these approaches in humans (NCT02800369, NCT03057912, NCT03226470, NCT03057912).

The same strategy was also performed to remove other viruses, such as herpes simplex virus (HSV) or hepatitis B virus (HBV), from the genome. Recently, Yin et al. (2021) proved that intracorneal delivery of lentiviral particles encapsulating Cas9/sgRNA targeting HSV could not only cure herpetic stromal keratitis in a mouse disease model, but also prevent HSV infection in a prevention model. Their meaningful data also boosted the initiation of the corresponding clinical trial (NCT04560790). HBV is one of the most important pathogens responsible for liver diseases. The HBV genome exists as a double-stranded covalently closed circular DNA (cccDNA), which can be targeted by gene-editing tools. Expression of ZFN pairs targeting the HBV genome resulted in specific DNA cleavage and inhibition of active HBV replication (Craddock et al., 2010; Weber et al., 2014). A similar strategy was also applied using TALEN (Bloom et al., 2013; Chen et al., 2014). Recently, CRISPR/Cas9 was also employed to achieve highly efficient HBV genome elimination (Dong et al., 2015; Ramanan et al., 2015). Notably, Dong et al. (2015) showed that injection of sgRNA-Cas9 plasmids via the tail vein reduced the levels of cccDNA and HBV protein in a mouse model carrying HBV cccDNA.

Another successful application of CRISPR/Cas for antiviral therapy was reported in 2014. Patient-derived cells from Burkitt’s lymphoma patient with latent Epstein-Barr virus infection showed dramatic proliferation arrest and a concomitant decrease in viral load after exposure to a CRISPR/Cas9 vector targeted to the viral genome (Wang and Quake, 2014). All these studies indicate that disrupting viral genes using gene editing is a promising strategy to treat infectious diseases.

Therapeutic gene editing: challenges and future directions

Advances in gene-editing technologies have laid the foundation for next-generation therapies to cure a wide range of genetic and nongenetic diseases. However, great effort in diverse fields is still required to enable safe, effective and affordable clinical translation. Here, we provide an overview of the existing challenges and potential approaches to surmount them. We also discuss issues remaining to be addressed in the future to enable gene-editing technology to fulfill unmet biomedical needs.

Challenges faced by current technologies

Immune responses elicited by gene-editing tools could influence the therapeutic effects

As reviewed in the above sections, gene-editing tools can introduce double-stranded breaks and perform precise genetic modifications in the genome by removing, replacing, or adding pieces of DNA at targeted sites. It is worth
highlighting that the majority of the enzymatic parts of these tools are derived from nonhuman species. For example, Cas proteins originate exclusively from bacteria, archaea or phages (Al-Shayeb et al., 2020; Makarova et al., 2020). Hence, the problem of immunity against exogenous proteins could not be avoided. On the one hand, preexisting neutralizing antibodies, along with antagonistic T cells against commonly used Cas nucleases, were found in the human body (Charlesworth et al., 2019; Wagner et al., 2019). Thus, the therapeutic efficacy may be compromised. Theoretically, these humoral and cellular immunity obstacles may be bypassed by exploiting new Cas orthologs from non-human-related microbes (Moreno et al., 2019) or by modifying the antigen epitope of the Cas proteins used if possible. On the other hand, adaptive immune responses against various components of CRISPR therapeutics could also be elicited. The effect of this problem is even more obvious when multiple dosing of gene-editing tools is needed. For example, Cas13-mediated RNA editing might require repeated dosing for long-lasting, effective transcriptional repression. In this situation, the therapeutic effects of the second dose or later doses could be impaired by the antibody produced following the first dose. In this case, using different Cas orthologs in each dosing to possibly evade adaptive immune responses might be one potential solution (Moreno et al., 2019).

Current gene-editing technologies can elicit unintended DNA or RNA modifications

The strong efficacy of existing gene-editing tools is usually accompanied by unintended DNA or RNA modifications. Taking the CRISPR system as an example, DNA recognition of the Cas-sgRNA complex cannot guarantee 100% specificity, as mismatches or other subtle changes in the target DNA can be tolerated (Hsu et al., 2013; Kleinstiver et al., 2016b; Strecker et al., 2019a; Tsai et al., 2015). In addition, scrambled trans-cleavage activity of Cas12 or Cas13 might occur following the cis-cleavage of targets (Abudayeh et al., 2016; Chen et al., 2018; East-Seletsky et al., 2016). Other proteinic components, such as the deaminase of base editors, could also cause nonspecific genome- and transcriptome-wide off-target deamination (Doman et al., 2020; Grünewald et al., 2019a; Grünewald et al., 2019b; Zhou et al., 2019a). More seriously, aside from off-target effects, nuclease-based gene editing can cause undesired on-target alterations in the genome, such as large elongated DNA deletions around the on-target DSBs (Adikusuma et al., 2018; Kosicki et al., 2018; Zuccaro et al., 2020) and changes to the chromosome structure, such as inversions or translocations (Frock et al., 2015; Maeder et al., 2019). To diminish these unintended gene editing outcomes, researchers have made great efforts to optimize these tools (Kim et al., 2019). However, to a certain degree, sacrificing on-target efficacy seems unavoidable for engineered tools (Schmid-Burgk et al., 2020). Recently, one work showed that AsCas12a and LbCas12a induced no trans-cleavage off-target effects in mouse embryos, possibly due to the low concentration of genomic DNA in the cell nucleus (Wei et al., 2021). This result may partially ease our anxiety about the adverse effects of Cas nucleases and indicate that unintended gene editing in vivo might be distinct from that detected at the cellular level. In any case, systematically evaluating the gene-editing outcomes of the therapeutic cargo is required before clinical treatment. Importantly, the safety issues mentioned above are largely influenced by the delivery approach, which we will discuss in the next section.

An important therapeutic application related to off-target issues is performing allele-specific gene editing to treat heterozygous dominant genetic diseases. It is crucial to prevent off-target editing at normal gene sites while efficiently disrupting the pathogenic allele. A suitable PAM sequence may not be located near the mutated sites for DNA editing (Li et al., 2018a). However, even when a canonical PAM exists, the mutations might not be enough for a 20-nt spacer to distinguish the two alleles (Li et al., 2018a). The rational design of spacers may partially solve these problems, for example, by using truncated or tuned sgRNA (Li et al., 2018a). A high-fidelity Cas enzyme to perfectly distinguish one-nucleotide difference between the wild-type and gain-of-function mutant allele is highly desired for this application. Nevertheless, this strategy is helpless when facing some complicated mutation types, for example, the expansion of repeated sequences (Rodriguez and Todd, 2019; Rudich and Lamitina, 2018). It seems that this problem may be addressed by RNA editing (Batra et al., 2017), as unproportioned RNA knockdown of transcripts from the two alleles can be accepted.

In vivo delivery of gene-editing tools

Benefiting from quality-controllable cell engineering with highly efficient biomacromolecule delivery methods such as electroporation, ex vivo genome editing has achieved impressive milestones in clinical trials (Esrick et al., 2021; Frangoul et al., 2021). However, compared with ex vivo gene editing, in vivo gene editing has advanced relatively slowly. One of the main contributing factors is the considerable complexity of our human body.

Therapeutic delivery vectors can be generally assigned into two distinct categories, viral and nonviral vector systems, according to their biological characteristics. AAV has become one of the most promising and commonly used viral vectors for gene therapy due to its high infection efficiency, broad tissue tropism, and low immunogenicity (Wang et al., 2019a; Wang et al., 2020a). However, the limited DNA packaging capability of AAV prevents the efficacious delivery of the commonly used SpCas9 nuclease and base and...
prime editors. Endeavors to discover smaller natural Cas nucleases or to evolve minimal Cas variants are an important need (Ran et al., 2015; Shams et al., 2021).

The wide application of AAV vectors for gene therapy also benefits from the resulting long-term gene expression. However, for gene-editing tools, transient expression is highly favorable because a long duration of efficacy can increase the safety risks mentioned above. From this perspective, it might be suboptimal to choose AAV to deliver gene-editing tools. However, a recent study reported that in vivo base editing in hepatocytes using SaCas9 KKH-CBE delivered by dual AAVs had no detectable off-target effects at either the RNA or DNA level (Villiger et al., 2021). The authors attributed this phenomenon to the low expression level of CBE in vivo due to the dual AAV strategy. Thus, it might be acceptable to carefully design the AAV vector to minimize the expression of its therapeutic cargo while maintaining its treatment efficacy.

On the other hand, nonviral vectors, especially nanoparticles, are highly suitable for the transient expression of the delivered cargos. A comprehensive summary of nonviral delivery strategies can be found in another review (Xu et al., 2021b). mRNAs commonly serve as therapeutic cargos for nanoparticles. The advantages of using nanoparticles to deliver mRNA-expressed gene-editing proteins are obvious. First, large base editors, prime editors, or multiple regulators can be introduced in mRNA format and packaged in nanoparticles to achieve efficient genome modifications (Song et al., 2020; Villiger et al., 2021). Moreover, some commonly used nanoparticles, such as lipid nanoparticles (LNPs), can be engineered with good manufacturing practices (GMPs) to ensure large-scale production and high quality (Kaczmarek et al., 2017). However, we should also note the limited tissue specificity of nanoparticles. Taking LNPs as an example, the predominant target site of most LNPs upon systemic delivery is usually restricted to the liver (Akinc et al., 2010). Although much effort has been made to engineer LNPs for broader tissue tropism (Samaridou et al., 2020), more information is needed on the mechanisms of the interactions between LNPs and targeted tissues.

The advantages of existing viral and nonviral vectors can be combined for an improved delivery strategy. One such strategy packaged mRNA in lentivirus virions (Ling et al., 2021). The authors further attached an MS2 recognition stem loop to the mRNA to mimic the package signal of the long terminal region (LTR), mediating an effective interaction with the capsid protein-fused MS2 protein. In this way, the high efficiency of transduction and transient expression of the two systems are combined together. This promising delivery method has been used to cure wet age-related macular degeneration and virus-induced herpetic stromal keratitis in a mouse model (Ling et al., 2021; Yin et al., 2021).

Future directions

Thanks to today’s powerful technologies, a major step forward was taken toward curing various diseases. However, patients still have unmet medical needs, which continues to drive scientists to develop novel gene-editing tools and therapeutic strategies. In this section, we will consider prospective future directions of gene-editing technology and discuss several tools with great potential to become novel therapeutic platforms.

Manipulating the human genome at a larger scale: correcting larger genomic variations

Nuclease-based genome editing may be used to correct mutations in many genetic diseases, especially HDR strategies, which can theoretically correct any genome alteration. In fact, this technology has been developing relatively slowly due to its extremely low efficiency in nonmitotic somatic cells (Cox et al., 2015; Zheng et al., 2014). New alternative tools, including base editors and prime editors, were developed to meet the need to correct mutations. These two editors can precisely and efficiently correct gene alterations such as point mutations, small genomic deletions, or insertions (<60 bp) (Anzalone et al., 2019; Gaudelli et al., 2017; Komor et al., 2016). However, it is very difficult for them to correct larger genome variations (>100 bp), such as genetic deletions, insertions, duplications, or inversions (insertions plus deletions), which cause many types of severe genetic diseases (Landrum et al., 2014). Although multiple guides can be employed to direct Cas nucleases to induce deletions or inversions of large DNA fragments (Maeder et al., 2019), undesired byproducts occur, increasing safety risks. It is worth noting that several works reported on bioRxiv re-purposed the prime editor to delete large DNA fragments using dual peg-sgRNAs (Choi et al., 2021; Jiang et al., 2021). However, the efficacy of this approach needs to be improved.

Accordingly, new technologies enabling manipulation of the human genome on a larger scale with high efficiency and precision are an important need. Here, we regard recombinase as a candidate, some types of which have already been widely applied in human cells to create large gene inversions and deletions with high efficiency and precision (Meinke et al., 2016; Van Duyne, 2015). One significant problem is that the recognizable DNA sequence for recombinase is highly restricted, making it difficult to apply to the natural human genome. In addition, engineerable sites on the human genome conforming to the canonical recognition model of recombinase are extremely limited (Lansing et al., 2020b), leaves little space for directed evolution or rational design. Fortunately, heterodimers of recombinases can be employed to recognize targets with asymmetric half-sites (Lansing et al., 2020b). Increasing the number of sites on the human genome can be chosen as templates for protein
engineering. Encouragingly, this strategy has been used successfully to correct genomic inversions in a gene that encodes factor VIII causing hemophilia A in human cells (Lansing et al., 2020a).

A more general strategy for curing genetic diseases: from gene scissors to gene glue

Precisely correcting gene mutations in situ is ideal for curing genetic diseases. However, the dysfunction of one gene can be attributable to hundreds of different genetic alterations, which makes such personalized correction unaffordable in terms of time and expenses. Thus, developing a more general strategy to treat diseases caused by different genotypes is highly desirable. One elegant idea is to insert an extra copy of a gene in the human genome regardless of the existing aberrant one. A cDNA could be precisely integrated behind a normal endogenous gene, or a complete gene cassette could be inserted into safe harbors (Barzel et al., 2015; Wang et al., 2019b). It is unavoidable to refer again to HDR, which may have the ability to achieve this goal. However, as discussed above, the working mechanism of HDR relies heavily on the endogenous DNA repair machinery, which causes low efficiency in nonmitotic cells. Nevertheless, we should emphasize the availability of HDR when integrating gene fragments into the genomes of mitotic cells such as hepatocytes or stem cells (Barzel et al., 2015; Martin et al., 2019; Yin et al., 2014; Yin et al., 2016).

Transposase can integrate large DNA fragments into the genomes of diverse cell types with high efficiency (Hickman and Dyda, 2016; Ivics et al., 2009). In contrast to that of recombinase, the recognition sequence of the commonly used transposase is quite simple, which leads to random integration of the donor in the human genome. Thus, efforts should be made to restrict the function of transposase to unique sites in the human genome. One simple idea is to conjugate transposase with specific DNA recognition modules, but the efficiency and specificity of these systems remain to be further optimized (Feng et al., 2010; Kovač et al., 2020; Luo et al., 2017; Owens et al., 2012; Owens et al., 2013). In addition, we should note that the fused specific DNA recognition modules concentrated the transposase near the targeted sites, whereas the intact transposase domain still performed its genome-wide functions. This obstacle can potentially be addressed by two newly discovered CRISPR transposase systems: CRISPR-V-K and CRISPR-I-F (Klompe et al., 2019; Strecker et al., 2019b). Both of them enable efficient and programmable targeted integrations in bacterial genomes (Klompe et al., 2019; Strecker et al., 2019b; Vo et al., 2021). More work should be focused on elucidating the efficacy of these two systems in mammalian cells in the future.

Compliance and ethics

The author(s) declare that they have no conflict of interest.

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