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Adenine base editing as a promising therapy for cardiovascular diseases

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Abstract

Cardiovascular diseases (CVDs) are the leading causes of human death worldwide. Genetic variants serve as the major risk factor for CVDs, with limited therapeutic interventions in clinical practice. The recent surge of genome editing technologies offers the hope to correct genetic variants and to cure genetic diseases. Among the diverse genome editing tools, adenine base editors (ABEs) exhibit high efficiency, high specificity, and low off-target effects, successfully entering a clinical trial and demonstrating the tremendous potential to transform modern cardiovascular therapy. In this review, we summarize the basic knowledge about ABE, showcase three hallmark studies using ABE to ameliorate or treat CVDs in experimental animals, and lastly discuss about the key technical concerns that should be addressed to achieve the full potential of ABEs in the future.

Keywords: Adenine base editor; Cardiovascular disease; Gene therapy

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1. Introduction

Cardiovascular diseases (CVDs) are the leading causes of morbidity and mortality worldwide. Genomic variants, often in the form of single nucleotide variants (SNVs), are one of the major causes of CVDs[1-3]. In the past decade, many CVD-associated SNVs were discovered, thanks to the advancement in high-throughput sequencing technologies[4]. However, effective therapies for these diseases remain absent.

The recent emergence of the genome editing technology has provided an unprecedented opportunity to treat CVDs. This technology was derived from the clustered regularly interspaced short palindromic repeats (CRISPRs) system in prokaryotes[5,6]. The CRISPR repeats in the prokaryotic genome encode an array of small non-coding RNA called the CRISPR RNA (crRNA). crRNA together with trans-activating crRNA (tracrRNA) was later engineered to form a single guide RNA (sgRNA)[7], which can direct the CRISPR-associated (Cas) nucleases, such as Cas9, to bind to a specific DNA sequence that is base-paired by the crRNA. Next, the nuclease locally digests the DNA and creates a DNA

double-stranded break (DDB), which can be repaired through either non-homologous end jointing (NHEJ) or homology-directed recombination (HDR)[6].

HDR-based genome editing can precisely write DNA sequences at the will of the scientists by providing a template DNA donor. However, the application of HDR is limited by its low editing efficiency, which is further complicated by the small nucleotide insertions and deletions (Indels) that are simultaneously created by NHEJ reaction[5]. Indels usually outnumber the HDR products among the genome-edited cell population, depositing unwanted frame-shifting mutations, so conventional CRISPR/Cas9 genome editing is usually more useful in gene silencing applications, while more efficient and precise genome editing tools, such as the base editors (BEs), are necessary for the correction of disease-causing genetic variants.

BEs are initially derived from the Cas9 nickase (nCas9)[8], which is a Cas9 mutant that only cuts one DNA strand, greatly reducing DDB formation and the introduction of indels at the edited loci. BEs are constructed by fusing nCas9 with an engineered deaminase that preferentially catalyzes nucleotide conversions on DNA[8,9]. The two most widely used BEs are the cytosine base editors (CBEs)[8] and the adenine base editors (ABEs)[9]. In CBEs, nCas9 is fused to the cytidine deaminase APOBEC1 to catalyze the nucleotide conversion of cytidine (C) to uracil (U), which is next modified as a thymidine (T) by the endogenous DNA repair system. In ABEs, the tRNA adenosine deaminase (TadA) is engineered to enable adenosine (A) deamination into inosine (I), which is next converted to guanidine (G) (Figure 1).

Among the SNVs that are known to associate with human diseases, nearly half of them are mutated from the C·G pair to the T·A pair[9,10], falling into the situation in principle reversible by ABEs. ABEs also demonstrate simpler domain structures and lower off-target effects as compared to CBEs[11,12] (Figure 1). Thus, ABEs have become the most popular BE tools that are promising in translational medicine.

In this review, we navigate our focus on the key technical features of ABEs and introduce the recent landmark gene therapy studies for CVDs. We also discuss about the technical concerns on the road toward successful clinical applications.

2. Basic parameters for ABE therapy

2.1. Editing efficiency

The effort to fuse TadA to nCas9 to edit adenosine initially failed with no detectable edits[9], because natural adenosine deaminases usually function on RNA but not DNA[9]. Dr. David Liu's team solved this problem through a directed evolution experiment on TadA, uncovering mutations at or near the TadA D108 residue as the key modifications to enable ABE activity on DNA substrates[9]. Additional molecular evolutions and trials-and-errors in testing mutation combinations in TadA eventually lead to the TadA7.10 mutant as well as the corresponding ABE prototype called ABE7.10[9].

Because TadA and Cas9 were derived from prokaryotes, ABE7.10 requires additional modifications to adapt to the applications in mammalian cells. First, the codon usage in the ABE7.10 gene was modified by introducing silent mutations so that ABE7.10 was better translated into proteins by tRNAs preferentially expressed in mammals. The second adaption involved the addition of nuclear localization signals (NLSs), so ABE7.10 could be transported into the cell nucleus for genome editing in eukaryotes. After the initial codon optimization and NLS incorporation efforts in ABE7.10[13], these parameters were, further, optimized in following studies, eventually leading to a more robust ABE variant called ABEmax[13,14].

The wildtype TadA protein acts as a dimer. Unlike in bacteria cells, where the TadA in ABEs can pair with the endogenous TadA to facilitate genome editing, mammalian cells do not express TadA. To solve this problem, in ABE7.10 and ABEmax, an evolved TadA was fused in tandem with a wildtype TadA to allow intramolecular dimerization[9]. However, in following studies, additional molecular evolution led to the discovery of ABE8e and ABE8.20, in which the TadA mutant can function as a monomer and exhibit even higher editing activity[15,16] (Table 1).

2.2. The scope of the editable adenines

Several factors determine if a specific adenosine is suitable for ABE. First, this nucleotide must be positioned within an "editing window" defined by the position of the sgRNA. In the CRISPR/Cas system, sgRNAs must be placed next to a protospacer adjacent motif (PAM), which is determined by the intrinsic property of the Cas protein. For example, the PAM sequence of a wildtype SpCas9 protein is NGG (N means any nucleotide)[17,18], while natural SaCas9 uses NNGRRT (R means A or G) as the PAM sequence[19]. The PAM sequence determines the location, where the CRISPR/Cas9 system unwinds the DNA to form R-loop[20], which exposes the single-strand DNA in the editing window to TadA for the deamination reaction (Figure 1).

In addition to PAM, the editing window of ABEs is also determined by their enzyme activity and their structural features. Usually, the editing windows of ABEs are 4-5nt wide. With the use of more robust TadA variants, the editing windows of ABEs can be broadened[15,16]. However,

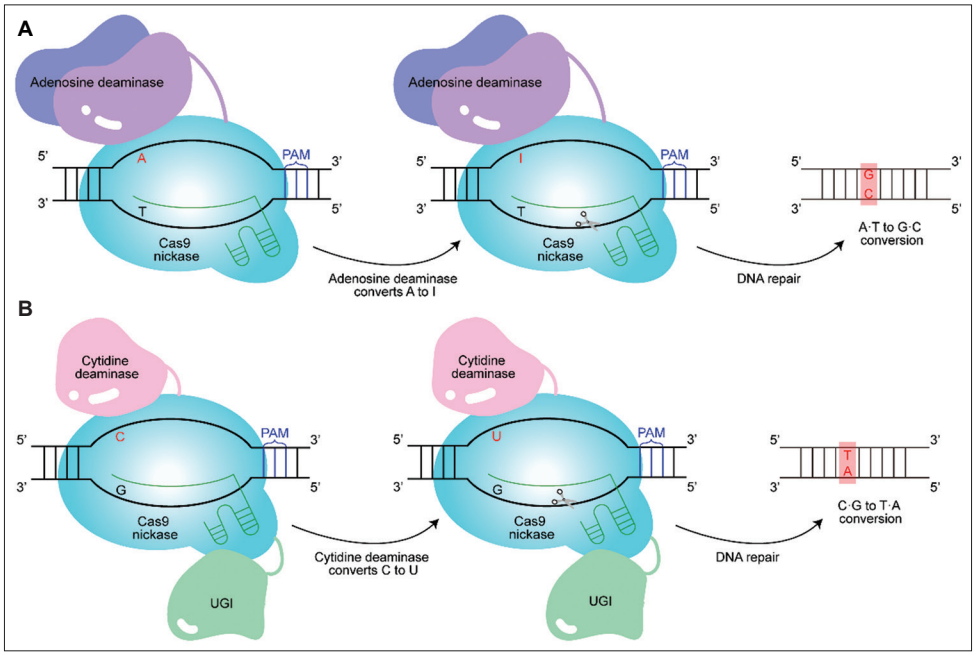


Figure 1. The working model of adenine base editing and cytosine base editing. (A) The adenine base editor (ABE) consists of adenosine deaminase TadA and Cas9 nickase (nCas9). SgRNA guides TadA-nCas9 to target the genomic DNA sequence by complementary base pairing. nCas9 unwinds DNA and exposes adenine on a single DNA strand for TadA-based editing. nCas9 also cleaves the non-edited DNA strand to facilitate DNA repair. Adenosine deaminase converts adenosine (A) to inosine (I), which is recognized as guanosine (G) in DNA repairing. Consequently, the ABEs mediate DNA base editing to convert A·T to G·C. (B) The cytosine base editors (CBEs) consist of cytidine deaminase APOBEC1, Cas9 nickase (nCas9), and uracil DNA glycosylase inhibitor (UGI). SgRNA guides APOBEC1-nCas9-UGI to target the genomic DNA by complementary base pairing. nCas9 unwinds DNA to generate an R loop and expose cytidine for APOBEC1-based editing. nCas9 cleaves the non-edited strand to facilitate DNA repair. Cytidine deaminase converts cytosine (C) to uracil (U), which is recognized as thymine (T) in DNA repairing. UGI inhibits uracil N-glycosylase (UNG) to prevent the reversal of U·G mismatch back to C·G base pair. Consequently, the CBEs mediate DNA base editing to convert C·G to T·A.

while a broader editing window means the increased likelihood of editing the target adenosine, it will also increase the bystander effect by introducing unintended editing of other nucleotides, particularly other adenosines, within the same window.

A major strategy to broaden the scope of editable adenosines is to fuse TadA with a Cas effector protein that uses different or less restrictive PAM sequences. These Cas orthologs can be discovered from the wild microbiome. Good examples include the wide variety of Cas9 and Cas12 family members[21,22]. Cas proteins can also be engineered to alter their PAM sequences. Successful examples include SpCas9 variants SpCas9-VRQR[23], which recognizes NGA as the PAM. Other commonly used Cas9 variants include SpCas9-NG[24] and SpG[25], which both use NGN as the PAM. Strikingly, the recently developed SpRY mutant uses the NRN (R means A or G) or NYN (Y means C or T) PAM and almost completely circumvents the PAM restraints[25].

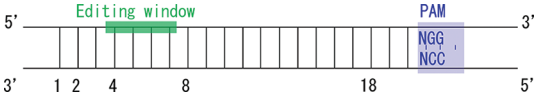
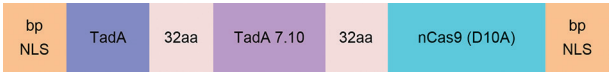
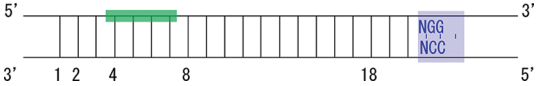
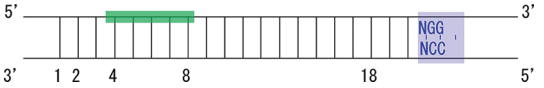
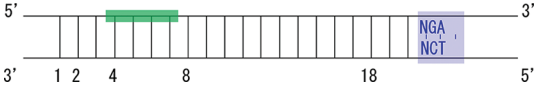
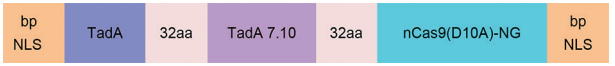
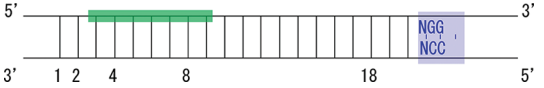
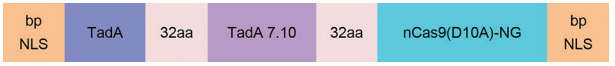
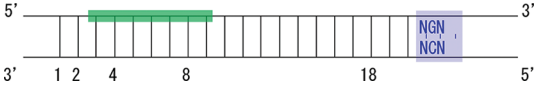
2.3. The gene delivery vector

To treat CVDs, the genome editing tools need to be effectively delivered to the cells that play a primary role in the disease. At present, the most successful and popular gene delivery vectors of ABEs include recombinant adeno-associated virus (rAAV) vectors and lipid nanoparticle (LNP) vectors.

rAAVs are viral particles that were engineered from the adeno-associated virus of the dependovirus genus of the parvoviruses[26,27]. An rAAV particle is composed of a protein capsid and an enclosed single-strand DNA of less than ~5kbp. As a non-pathogenic virus, rAAV can effectively transduce a number of organs, including the heart, with relatively low immunogenicity and toxicity. As of January 1, 2023, six rAAV-based gene therapy drugs have been federally approved for the treatment of different diseases, building an excellent safety, and effectiveness record for this new drug format[28]. The trademark names of these drugs are Glybera, Luxturna, Zolgensma, Upstaza, Roctavian, and Hemgenix.

The coding sequence of ABE7.10 is about 5.4 kbp in length, beyond the packaging capacity of rAAV vectors[9]. The mainstream solution of this problem harnesses the split intein system to allow two parts of the proteins to trans-splice into a full-length protein[29]. Therefore, ABE can be split into two halves, each being delivered by two separate

Table 1. Representative ABE variants

ABE variants	Base editor architecture	Editing window and PAM	References
ABE7.10			[9]
ABEmax			[12]
ABE8e			[14]
ABEmax -VRQR			[57]
ABE8.8			[15]
ABEmax -NG			[13]

NLS: Nuclear localization signal, asa: Amino acid, bpNLS: bipartite NLS. In all panels, the ABEs editing windows are shown in green and PAM sequences in blue

rAAV vectors[30]. Alternatively, the recent engineering of smaller Cas proteins and the more compact designs of the rAAV vector has enabled ABE delivery using a single rAAV particle[31].

Another well-established tool for *in vivo* ABE delivery is the LNPs, which are nanoscale semi-solid particles that are assembled by four types of lipids, namely, cholesterol, phospholipids, ionizable lipids, and PEGylated (PEG means polyethylene glycol) lipids. The ionizable lipid can undergo a pH-dependent charge conversion and allow mRNA encapsulation into LNP[32]. On intravenous administration, conventional LNPs deliver nucleic acids primarily to the liver. The recent development of novel lipid formulas allows LNPs to target the lungs, the spleen, and some other organs[33].

Since the first U.S. Food and Drug Administration (FDA) approval of LNP drugs in 2018[34], LNPs have drawn tremendous attentions from both biotechnological and pharmaceutical researchers. LNPs have demonstrated a great safety record largely, because the lipid components can be quickly metabolized and cleared from the body. As the major vector for COVID-19 mRNA vaccine, the LNP technology and industry are both rapidly growing and maturing[32]. LNPs have recently carried the first ABE drug for CVDs into a clinical trial (NCT05398029), holding the great promise to facilitate ABE-based treatment of more human diseases.

3. Landmark studies of ABE therapy for CVDs

3.1. Hutchinson-gilford progeria syndrome

One of the first evidence demonstrating the effectiveness of ABE in gene therapy involves the Hutchinson-Gilford Progeria Syndrome (HGPS)[30]. HGPS is a rare disease with whole-body premature aging phenotypes. Among these phenotypes, vascular malformation and dysfunction are most critical as these patients usually die of atherosclerosis and heart attacks in their teens[35]. Therefore, here we treated HGPS as a special type of CVD.

HGPS is commonly caused by a heterozygous *LMNA* c.1824 C>T/p.G608G mutation. This mutation activates a cryptic splicing site in the gene and aberrantly produces a splicing variant protein called progerin[36-38]. Despite much effort to reduce the toxic effects of progerin, particularly with the development of the FDA-approved farnesyltransferase inhibitor drugs[39], the patients can only survive for another 2–3 years.

The *LMNA* c.1824 C>T mutation falls into the SNV category that is editable by ABE. To test this idea, Dr. David Liu's team firstly used a lentiviral vector to deliver the ABEmax-VRQR base editors to treat fibroblasts that are derived from HGPS patients. After a puromycin selection for virus-transduced cells, the authors observed ~84% correction of the pathogenic mutation, substantially reduced progerin expression, and the ameliorated nuclear shape phenotypes[30].

Next, the same team designed a dual-AAV system to deliver ABEs to a transgenic HGPS mouse model that constitutively expresses the human progerin (Figure 2A). A single intravenous injection of these AAVs resulted in variable editing efficiencies (10 – 60%) among the heart, the quad, the liver, the aorta, and the bones. Strikingly, this single-dose treatment was sufficient to reduce the loss of vascular smooth muscle cells and the periadventitial thickening of the aorta, which are key pathological features of HGPS. This ABE treatment also increased the median lifespan of the mouse model from 215 to 510 days, approaching the old age of healthy mice[30]. Therefore, AAV-mediated ABE treatment might potentially be a permanent cure for HGPS in the future.

3.2. Inherited hypertrophic cardiomyopathy

In addition to HGPS, hypertrophic cardiomyopathy (HCM) is another CVD that would potentially benefit from ABE treatment. Unlike HGPS, which is a very rare disease with a prevalence of 1 in 20 million people, HCM is the leading cause of cardiac sudden death in people younger than 35-years-old[40]. HCM is featured by the excessive thickening of myocardium and the hypercontractile phenotype of cardiomyocytes[41]. SNVs in genes coding sarcomere proteins, particularly *MYH7* and *MYBPC3*[42], are the major causes of HCMs. Despite the recent development of cardiac myosin inhibitors[43-45] as breakthrough drugs for HCM, their application is usually limited to a subgroup of HCM patients, and their therapeutic effects are far from being satisfactory. Therefore, it is critical to develop a new approach to treat this disease.

Dr. Feng Lan's group performed the first proof-of-concept study to test the ability of ABE to treat HCM in mice[46]. They created a clinically relevant mouse model carrying the HCM pathogenic MYH6-R404Q/+ mutation (*Myh6* c.1211C>T) and validated its pathogenic role in HCM. Then, they microinjected ABEmax-NG mRNA and sgRNA into the mutant zygotes, allowing the embryo to develop to birth, and then genotyped the animals to evaluate the effect of the ABE (Figure 2B). Their results demonstrated that the overall editing efficiency is 91% on the *Myh6* c.1211C>T loci among the mutant embryos. The genetically corrected mice showed normal heart weight, less fibrosis, orderly arranged myofilaments, and normal left ventricular wall thickness, effectively preventing the HCM phenotypes in the R404Q/+ mice.

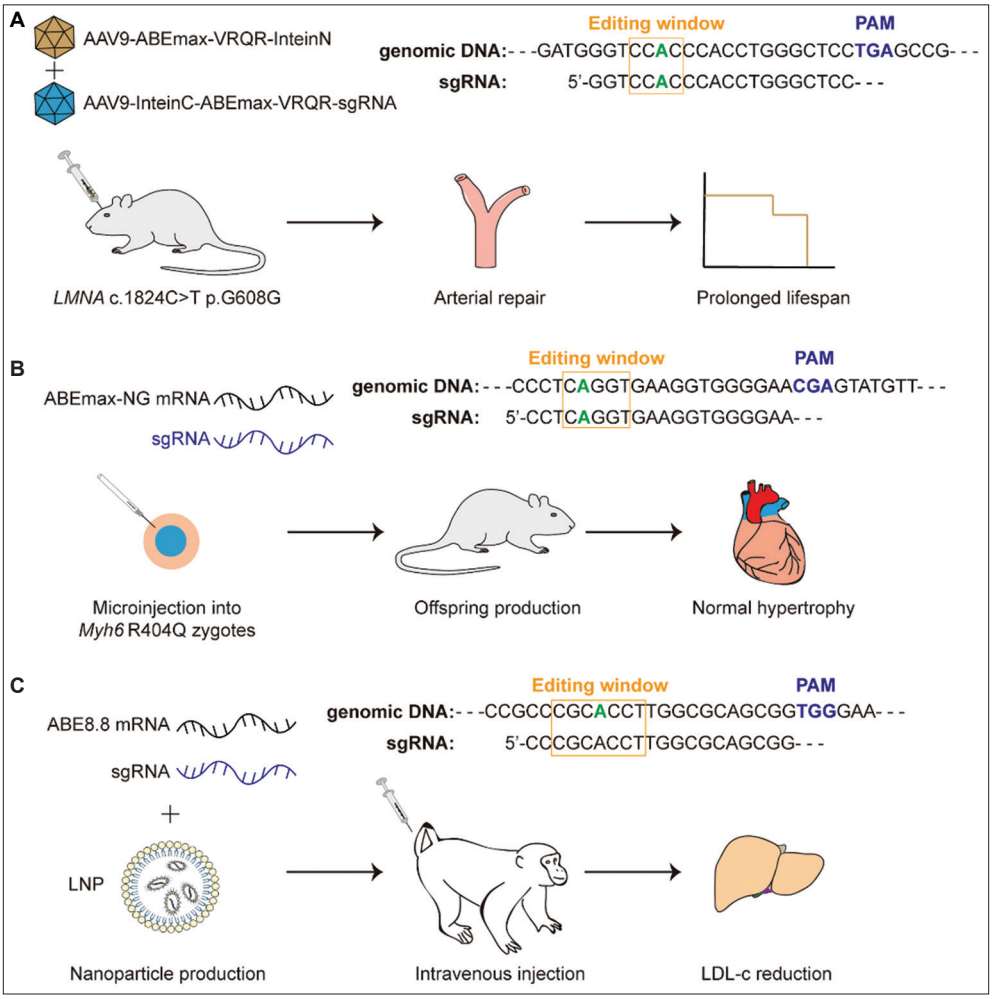


Figure 2. Three hallmark studies of ABE-based therapy for CVDs. (A) ABE gene therapy for HGPS in mice. Dual AAV9 expressing ABEmax-VRQR and sgRNA were retro-orbitally injected into HGPS mouse models, which resulted in the prevention of arterial damages and the increase of lifespan. (B) ABE gene therapy for HCM in mice. ABEmax-NG mRNA and sgRNA were co-microinjected into the Myh6-R404Q/+ zygotes, which prevented the development of HCM. (C) ABE gene therapy for hypercholesterolemia and atherosclerotic CVDs. The LNPs that carried ABE8.8 mRNA and sgRNA were delivered into cynomolgus monkey by intravenous injection. The LDL-C was reduced significantly. In all panels, the orange box depicts the editing window with the PAM sequences in blue.

Although zygotic genome editing provides a powerful technique for the proof of concept, germline gene therapy is apparently difficult in clinical practices and would raise serious ethical issues particularly when genome editing is performed[47]. As a test for somatic gene therapy, Dr Lan's group also established a dual-AAV system to deliver ABE into embryonic day-16 mutant fetuses. In contrast to zygotic editing, the AAV system only corrected 25.3% of the pathogenic mutation. This editing efficiency was further reduced if AAV was injected at a later time point, suggesting that the performance of ABE heavily depends on the developmental stage of the heart[46]. The authors argued that the success of base editing required active DNA replication or cell cycle. Thus, mature cardiomyocytes, as a terminally differentiated cell type[48], might be difficult to edit postnatally. This problem might be the major bottleneck in the efforts to treat inherited cardiomyopathy by ABE.

3.3. Hypercholesterolemia and atherosclerotic CVDs

Both HGPS and HCM are diseases with clear pathogenic mutations. Such SNVs are often rare, and it is not practical to develop a different ABE drug for each individual SNV. In addition to the correction of missense mutations, ABE can also be harnessed for gene silencing, which greatly expands the application of ABE in gene therapy. Below is an example that has pushed ABE to a clinical trial (NCT05398029), providing an exciting opportunity to treat hypercholesterolemia and to prevent atherosclerotic CVDs, the leading cause of death worldwide[49].

Atherosclerotic CVDs are caused by the narrowing and hardening of the artery walls and the formation of plaques[50]. The elevation of low-density lipoprotein cholesterol (LDL-C) in blood is a major cause of atherosclerosis[51]; thus, reducing LDL-C is a well-accepted strategy to treat atherosclerotic CVDs. PCSK9 is a plasma protein that accelerates the removal of LDL receptors from the surface of hepatocytes, preventing the uptake and clearance of blood LDL-C[52]. Most importantly, non-sense mutations of *PCSK9* in the human population are associated with lower LDL-C levels and a significantly reduced risk of coronary heart disease[53,54], without causing other serious abnormalities. Thus, *PCSK9* has served as a classic therapeutic target to reduce LDL-C for many years.

PCSK9 is mainly expressed and secreted by the liver; thus, liver-targeted gene delivery vectors, such as LNPs, provide ideal tools to genetically manipulate *PCSK9 in vivo*. In 2021, Musunuru *et al.* designed an elegant ABE system to edit the 5' splice donor sequence in *PCSK9* intron 1, disrupting pre-mRNA splicing, and depositing a premature stop codon that ablates *PCSK9* expression[55]. They used LNPs to deliver ABE8.8 mRNA and the sgRNA targeting *PCSK9* into cynomolgus monkeys (Figure 2C) and detected about 70% editing rate specifically in the liver. This one-time treatment results in almost complete elimination of blood *PCSK9*, as well as over 50% reduction of LDL-C for up to 8 months, suggesting the capacity to lower LDL-C with one shot for life[55]. In July 2022, this investigational drug has been dosed in the first human in a phase 1b clinical trial, as a potential treatment for heterozygous familial hypercholesterolemia[56]. If successful, this game-changing therapy is expected to be repurposed for other forms of atherosclerotic CVDs in the future.

4. Future challenges

4.1. Identifying and expanding the editable loci

The first step in designing an ABE study is to determine if the target adenosine is editable. At present, this task is challenged by several factors including the availability of a PAM sequence at the appropriate position, the presence of other adenosines in the editing window that might result in unwanted bystander effects, as well as the sequence and chromatin neighborhood that might undermine the editing efficiency.

To overcome these limitations, huge progress has been made to introduce new Cas proteins with distinct or less restrictive PAM sequences[17,24,25,57] in to ABEs. For example, a recent study equipped TadA8e with multiple distinct Cas9 variants and collectively offered editability to about 82% adenosines in the human genome[31]. TadA was further engineered to change the editing windows[15,16,58,59] or to enhance the position precision of ABE so that on-target editing can be achieved while reducing the bystander effects. In addition, machine-learning approaches have also been exploited to predict the outcome of a given ABE reaction *in silico*[60-62], greatly reducing the costs and efforts in the experimental exploration of a good ABE design for a specific application.

4.2. Increasing editing efficiency

The editing efficiency of ABE is firstly determined by the design of the ABE machinery. Codon optimization and the proper installation of nuclear localization sequences (NLS) have been shown to enhance the performance of ABE[13]. The modulation of the linker amino acids between TadA and the Cas9 nickase and the coupling of ABE with an uracil glycosylase inhibitor (UGI) could also improve editing efficiency[63]. Most importantly, the directed evolution of TadA has been shown as a powerful approach that continuously increases the editing efficiency of ABEs[9,15,16].

In addition to the intrinsic properties of ABE itself, its editing efficiency is also influenced by the availability and expression levels of ABE components in the cells, which is determined by the gene delivery methods. For example, LNP has be validated as a robust vector to deliver ABEs to the liver to achieve high editing efficiency[55]. With the recent development of new formula, LNPs can also target the lungs, the spleen, and a couple of other organs[33], but whether these new tools will lead to robust editing in these organs remains to be examined.

As compared to LNPs, AAV vectors have been demonstrated to permit base editing in more organs, but the editing efficiency is relatively low[30]. The small payload of AAV vectors has been the major limiting factor, so in the dual-AAV systems, cells can be edited only when both AAV vectors transduce the same cell. With the development of more compact ABE tools and the careful design of AAV vectors, recent studies have started to report all-in-one AAV-ABE vectors[31], which indeed increased editing efficiency as compared to the dual-AAV systems. However, these vectors have pushed the AAV payload to the extreme, leaving little space for further modifications of these vectors.

4.3. Reducing the undesired editing

The precision of ABE reaction determines the safety of the relevant therapies. Thus, undesired editing by ABEs needs to be carefully monitored when developing new drugs. These unwanted editing can be grouped into two types, namely, the ones on the targeted site and the ones on the

off-target site (Figure 3). An important form of unwanted editing on the target site is the indels. Because Cas9 nickases are used in ABE, its likelihood to introduce indels is very low. Among the key studies of ABE therapy for CVDs, the indel rate has be reported as 0.2% or lower[30,46,55].

As mentioned previously, the more problematic form of unwanted editing on the target sequence involves the bystander effect (Figure 3A). The three hallmark studies using ABE for CVD therapy all cleverly chose the target sites with only one adenosine in the editing window, circumventing this problem[30,46,55]. However, for most other diseases, it will be inevitable to edit an adenosine near other adenosines. Fortunately, a recent study reported a new version of ABE called ABE9, which exhibited a narrow editing window of only 1-2 nucleotides[59]. By carefully choosing Cas9 variants with less PAM restriction and designing sgRNAs to put only the target adenosine in the editing window, it is promising to drastically reduce the likelihood of bystander effects in the future.

The undesired edits of ABEs on nucleic acid sites distinct from the targeted site are often called the off-target effect (Figure 3B-D). Because TadA was originally an RNA deaminase, a major ABE off-target effect was found on RNA transcripts (Figure 3B), which was seemingly independent from Cas9 and sgRNA[64]. In addition, ABE can deposit unwanted edits on sites with 1-2nt mismatches to sgRNA (Figure 3C), which is known to be tolerated by Cas9[18,65,66]. Interestingly, ABEs can also exert the genomic off–target effect in a Cas9/sgRNA-independent manner (Figure 3D)[15,67]. This type of editing most likely happens in genomic loci that naturally unwind and expose single-strand DNA to the freely available TadA in the nuclei, such as during DNA replication and gene transcription.

Because off-target effects are induced by complicated mechanisms, the prediction and identification of off-target sites by a given ABE reaction are challenging (Table 2). The most convenient and fast methods to nominate off-target sites are through computational prediction. Although these methods usually work fine in predicting sgRNA-dependent off-target sites basing on sgRNA similarity[68,69], or when sufficient prior data are available for machine-learning based prediction[70,71], additional experimental validation is still necessary to avoid false-positive nominations. Off-target effects can also be assessed experimentally by either using purified genomic DNA[72-75] or through cell culture[76,77]. However, these methods still cannot fully characterize the off–target effects in animals or human bodies where ABE is used as a therapy. Whole genome sequencing (WGS) was a universal approach to detect the off-target effect both in cells and animal tissues[78]. The recent development of DISCOVER-seq (discovery of in situ Cas off-targets and verification by sequencing) and GUIDE-tag (GUIDE means genome-wide, unbiased identification of DSB) techniques provides promising tools to directly assess the off-target effects for *in vivo* genome editing[79,80]. However, whether these methods are sensitive

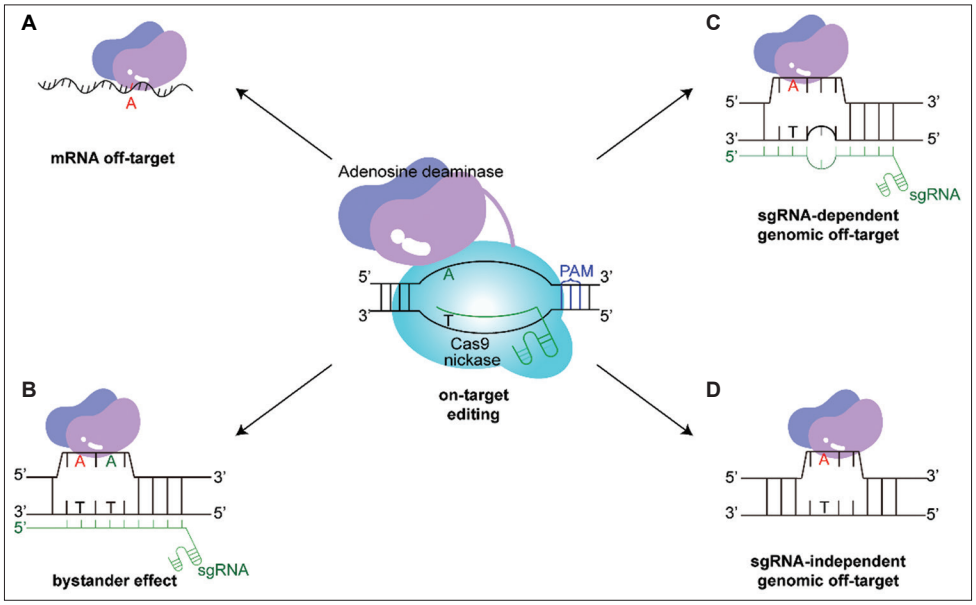


Figure 3. The types of undesired ABE editing. (A) Bystander effect. TadA edits other untargeted adenine in the editing window of the on-target site. (B) The off-target effect on mRNA. TadA modified adenine in mRNA independent of Cas9/sgRNA. (C) sgRNA-dependent genomic off-target effect. The sgRNA tolerates 1–2nt mismatches and guides ABE to modify an off-target site. (D) sgRNA-independent genomic off-target effect. TadA converts A to G in some genomic DNA sites independent of sgRNA or Cas9. In all panels, the undesired adenine edits are in red, while the target adenine in green.

Table 2. Representative methods to determine genomic off-target effects

Classification	Name	Advantage	Disadvantage
Computational prediction	Cas-OFFinder[67]	Simple, cheap and expedient	Biased toward sgRNA- dependent off-target sites. Still require further experimental validation.
	CasOT[68]		
	DeepCRISPR[69]		
	Elevation[70]		
In vitro detection on purified genomic DNA	Digenome-seq[73]	Unbiased and sensitive	Only applicable *in vitro*. With false positive and false negative hits due to the lack of intracellular factors.
	CIRCLE-seq[74]		
	SITE-seq[72]		
	EndoV-seq[71]	Specifically designed for ABE	
Label and capture in cell culture	IDLV capture[76]	Unbiased, sensitive and in living cells	Only applicable in cell culture. With false positive and false negative hits due to the lack of physiological relevance.
	GUIDE-seq[75]		
Direct detection in animal tissues	WGS[77]	Unbiased and suitable for clinical samples	Expensive and relatively low sensitivity
	DISCOVER-seq[78]	Unbiased and suitable for clinical samples	Relatively low sensitivity
	GUIDE-tag[79]	Unbiased and in animal tissues	Technically complicated and not suitable for clinical samples

Digenome-seq: *In vitro* Cas9-digested whole-genome sequencing, CIRCLE-seq: Circularization for reporting of cleavage effects by sequencing, SITE-seq: Selective enrichment and identification of adapter-tagged DNA ends by sequencing, IDLV: Integrase-deficient lentivirus, GUIDE-seq: Genome-wide, unbiased identification of DSBs enabled by sequencing, WGS: Whole genome sequencing. DISCOVER-seq: Discovery of *in situ* Cas off-targets and verification by sequencing

and accurate enough to evaluate ABE-induced off-target effects in gene therapy remain to be determined.

So far, two major approaches have been applied to reduce the off-target effects by ABE. The first method harnesses protein structure information or directed evolution technology to engineer more accurate ABE mutants. For example, arginine 153 (R153) within TadA was reported to mediate its RNA editing activity; thus, R153 deletion was implemented in ABEs to minimize its RNA off-target effects[81]. Importantly, the recently established ABE9 also drastically reduced both RNA off-target effects and Cas9-independent DNA off-target effects, in addition to the aforementioned impact on bystander effects[59].

Another plausible method to reduce the off-target effect works by controlling the duration of ABE expression. Because RNA exhibits a high turnover rate, RNA off-target effects will gradually taper off once the ABE stops expressing. Similarly, once the on-target DNA editing is accomplished, ABE activities should be terminated to avoid further accumulation of off-target edits in the genome. Based on this rationale, LNP vectors are more suitable than AAV vectors in delivering ABEs to the liver, as LNP-mediated ABE expression only persists for days, while AAV-mediated gene expression can last for years.

5. Conclusions

CRISPR/Cas9-based genome editing has revolutionized biomedical research, including cardiovascular research, in the past decade. With the emergence of more advanced genome editing tools such as ABE, therapeutic genome editing in human bodies has entered clinical trials and will likely become a reality in near future. In this review, we showcase the power of ABE in CVD therapy and recommend more cardiovascular researchers to embrace ABE as a new weapon to tackle CVDs.

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Conflict of interest

No potential conflicts of interest were disclosed.

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