The use of new CRISPR tools in cardiovascular research and medicine

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Abstract | Many novel CRISPR-based genome-editing tools, with a wide variety of applications, have been developed in the past few years. The original CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system was developed as a tool to alter genomic sequences in living organisms in a simple way. However, the functions of new CRISPR tools are not limited to conventional genome editing mediated by non-homologous end-joining or homology-directed repair but expand into gene-expression control, epigenome editing, single-nucleotide editing, RNA editing and live-cell imaging. Furthermore, genetic perturbation screening by multiplexing guide RNAs is gaining popularity as a method to identify causative genes and pathways in an unbiased manner. New CRISPR tools can also be applied to ex vivo or in vivo therapeutic genome editing for the treatment of conditions such as hyperlipidaemia. In this Review, we first provide an overview of the diverse new CRISPR tools that have been developed to date. Second, we summarize how these new CRISPR tools are being used to study biological processes and disease mechanisms in cardiovascular research and medicine. Finally, we discuss the prospect of therapeutic genome editing by CRISPR tools to cure genetic cardiovascular diseases.

The development of genome-editing technology has revolutionized biomedical research, particularly since the introduction of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) technology. This system was initially discovered as part of the immune response in bacteria[1] and was subsequently applied to eukaryotic genome editing[2–4]. The simplicity of the CRISPR/Cas9 system has made genome editing more accessible and easier than with traditional DNA editing techniques. As a genome-editing tool, the system consists of two basic components: an endonuclease Cas9, which cleaves DNA strands, and a single guide RNA (sgRNA), which contains a specific sequence to recognize the target DNA region of interest. Of note, the endogenous Cas9 system in bacteria has two RNA components (CRISPR RNA (crRNA) and trans-activating crRNA); the sgRNA in the CRISPR/Cas9 tool is artificially engineered from crRNA and trans-activating crRNA. When Cas9 and a sgRNA are delivered into cells, the resulting Cas9–sgRNA complex is directed to the target genomic site, where it generates double-strand breaks (DSBs) in the DNA. The DSBs are then repaired through endogenous DNA repair machineries, enabling gene knockout or knock-in[5].

In the past few years, the original CRISPR/Cas9 system has been repurposed for various applications that are not limited to editing DNA sequences in the genome[4]. Repurposing has been achieved by inactivating Cas9 (or other Cas nucleases) to prevent DSBs from being generated, either partially[6–8] or entirely[9,10], and fusing the inactivated Cas nucleases to a wide variety of effector proteins, which can be directed to the desired loci defined by sgRNAs without generating DSBs[4,10,11]. The expanded applications for this technology include gene-expression control, epigenome editing, editing of single-base nucleotides, RNA editing and live-cell imaging (FIG. 1). These new CRISPR tools expand the options for studying biological mechanisms and disease pathophysiology, such as with the use of in vitro and in vivo genetic models. In addition, multiplexed CRISPR screens, in which many genes are perturbed and functionally characterized simultaneously to identify causative genes in an unbiased manner, are becoming more common and could be performed using the new CRISPR tools. Moreover, these tools can be used therapeutically to treat diseases. For example, base editors are a promising option to achieve safer and more efficient genome editing than with the conventional CRISPR/Cas9 system to cure genetic diseases such as hyperlipidaemia[12,13]. In this Review, we survey the new CRISPR tools that have been developed to date and discuss how they can be used to study biological process and disease mechanisms related to the cardiovascular system. Furthermore, we appraise the

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Key points

- New CRISPR-based tools with various functions provide an increasing number of options to study disease mechanisms and cure genetic diseases.
- CRISPR screens to identify causative genes are becoming more common and can be combined with new CRISPR tools.
- New CRISPR tools, particularly base editors, have potential for therapeutic genome editing.
- In cardiovascular medicine, the focus of therapeutic genome editing is on the liver to reduce blood LDL-cholesterol levels.
- The high efficiency and specificity of new CRISPR tools could enable therapeutic genome editing of inherited cardiac and vascular diseases.
- Therapeutic genome editing requires further investigation of in vivo off-target effects and improved delivery methods.

potential therapeutic uses of the new CRISPR tools in cardiovascular medicine.

Emerging CRISPR tools

A growing number of novel CRISPR-based tools have been developed with a wide variety of applications not limited to conventional genome editing with non-homologous end-joining (NHEJ), microhomology-mediated end-joining (MMEJ) or homology-directed repair (HDR)[5] (see Supplementary Table 1 for a list of useful links for CRISPR tools). In the following sections, we focus our discussion on the main non-conventional CRISPR tools and discuss their potential uses in cardiovascular research.

Conventional CRISPR/Cas9 genome editing

To appreciate the differences between the conventional CRISPR/Cas9 system and the new tools, we must first understand the mechanisms of genome editing using the conventional method. First, the Cas9 protein is directed to the target DNA sites based on the spacer sequence in the sgRNA, where the target sites need to be followed by a short sequence called a protospacer adjacent motif (PAM). The Cas9 protein then hybridizes to the target sites and generates DSBs[2,3]. To achieve gene knockout or knock-in, the CRISPR/Cas9 system relies on endogenous repair pathways, including NHEJ, MMEJ and HDR[5] (FIG. 2a).

When DSBs are generated, the predominant repair pathway in most mammalian cell types is NHEJ, in which the ends of DSBs are directly ligated by endogenous repair machineries[5,14]. However, NHEJ is an error-prone process that often introduces small insertions or deletions (called indels) at the site of the junction. An indel in the coding sequence of a gene could induce a frameshift or a premature stop codon, leading to gene knockout. Another repair pathway of re-ligation is MMEJ, which involves the alignment of microhomologies (5–25 bp) at the ends and generates products in which the sequences between the microhomologies are removed[5,15]. As with NHEJ, MMEJ is also prone to introducing indels.

Alternatively, in the presence of a repair template, the DSBs can be repaired by HDR, which allows precise modifications to a desired sequence that is defined in the template[5,14]. The repair template can take the form of either double-stranded DNA, with two homology arms flanking the insertion sequence or single-stranded DNA oligonucleotides. Therefore, adding a donor template to the CRISPR/Cas9 system allows gene knock-in to be achieved at a desired locus. However, HDR has several weaknesses. First, editing is inefficient and typically requires the selection of cells that have been successfully edited. Second, this approach cannot be used in non-dividing cells such as cardiomyocytes because HDR is active only in the S or G2 phases of the cell cycle, unlike genome editing with NHEJ. For example, editing in murine cardiomyocytes showed limited knock-in efficiency because these cells are postmitotic[16,17].

Of note, the mechanisms described above are for typical CRISPR genome editing by Cas9. Other Cas nucleases (such as Cas12a; also known as Cpf1 (REF.[18])) also allow gene knockout and knock-in with a difference in their PAM sequences and cleavage patterns.

CRISPR with nuclease-deficient Cas

In the conventional CRISPR/Cas9 genome-editing system, the Cas9 proteins have two main features: binding to a specific DNA sequence when guided by a sgRNA and cleaving double-stranded DNA at the binding site. Inactivation of the cleaving feature allows for targeting of specific genomic loci without making DSBs[4,11]. This repurposing was first demonstrated by introducing mutations into the *Streptococcus pyogenes* Cas9 in both of its two nuclease domains, HNH and RuvC[9]. The resulting nuclease-deficient Cas9 (dCas9) is unable to cleave DNA but can still bind to the specific DNA sequence when guided by a sgRNA. Therefore, dCas9 can physically occupy the targeted genomic loci or allow the recruitment of effector proteins to the targeted loci without altering the DNA sequence. Namely, dCas9 can expand the applications of CRISPR technology beyond genome editing when fused with diverse effector proteins such as transcriptional repressors or activators and epigenetic modifiers[4,9,11]. Combined with a variety of effector proteins, nuclease-deficient Cas proteins are now widely used as the basis of new CRISPR tools such as CRISPR interference (CRISPRi), CRISPR activation (CRISPRa) and epigenome editors[11] (FIG. 2b,c).

In addition to nuclease-deficient dCas9, Cas9 'nickases' have been developed by mutating either of the two Cas9 nuclease domains[6–8]. As Cas9 nickases retain one active nuclease domain, they create a single-strand break rather than a DSB. For example, Cas9 D10A nickase, which has an inactivated RuvC domain, cleaves only the target strand. Conversely, Cas9 H840A nickase, which has an inactivated HNH domain, cleaves the non-target (PAM) strand. Cas nickases are used as the basis for several new CRISPR tools that require nicks such as base editors and prime editors[19,20] (FIG. 2d).

Gene-expression control

CRISPR-mediated gene repression, known as CRISPRi, was first demonstrated in *Escherichia coli* using dCas9 alone without effector proteins[9,21]. Under the guide of a sequence-specific sgRNA, a dCas9–sgRNA complex can occupy the targeted genomic loci or interfere with transcription initiation and elongation by disrupting transcription factor binding or by blocking RNA polymerase, respectively. To achieve increased

Non-homologous end-joining
(NHEJ). An endogenous cellular mechanism to repair double-strand breaks (DSBs), in which the ends at a cut site are directly ligated to each other.

Microhomology-mediated end-joining
(MMEJ). An endogenous cellular mechanism to repair DSBs, in which microhomologous sequences at both ends of a cut site are used to align the ends, resulting in the removal of the flanking region.

Homology-directed repair
(HDR). An endogenous cellular mechanism to repair DSBs, in which the sequence at a cut site is replaced by a sequence specified in a donor template, typically via homologous recombination.

Protospacer adjacent motif
(PAM). A short, specific DNA sequence (2–6 nucleotides) that follows the DNA sequence targeted by a CRISPR system and is required for a Cas nuclease to bind to the target region.

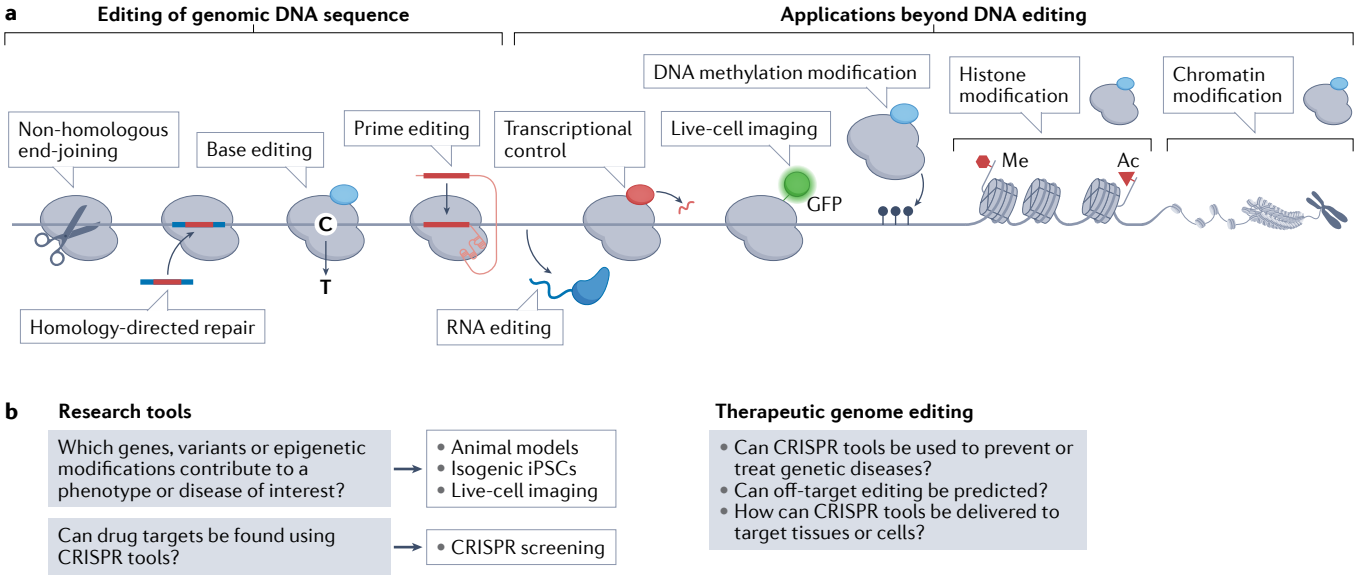


Fig. 1 | **New CRISPR technologies. a** | A wide variety of new CRISPR (clustered regularly interspaced short palindromic repeats) tools have been developed, ranging from precise base editing, transcriptional control, and epigenome editing to chromatin structure modification and live-cell imaging. **b** | As with the conventional CRISPR/Cas9 system, applications for the new tools are divided into two categories: as research tools to study biological mechanisms or disease pathophysiology and for therapeutic genome editing to prevent and treat diseases. Several important questions could be addressed using these new CRISPR technologies. Ac, acetyl group; C, cytosine; iPSC, induced pluripotent stem cell; Me, methyl group; T, thymine.

repression in mammalian cells, a Krüppel-associated box (KRAB) or four concatenated mSin3 interaction domains (SID4X) were fused to either the N-terminus or the C-terminus of dCas9 as effector proteins, and the fusion proteins (dCas9–KRAB or dCas9–SID4X) were able to knockdown endogenous genes in mammalian cells more efficiently than dCas9 alone[4,22] (FIG. 2b). CRISPRi tools are used in many fields, including cardiovascular medicine[23–29] (TABLES 1,2). For example, dCas9–KRAB was used to knock down *CAML2* in induced pluripotent stem cell (iPSC)-derived cardiomyocytes (iPSC-CMs) generated from a patient with long QT syndrome (LQTS), resulting in functional rescue of LQTS phenotypes such as prolonged action potential duration[24].

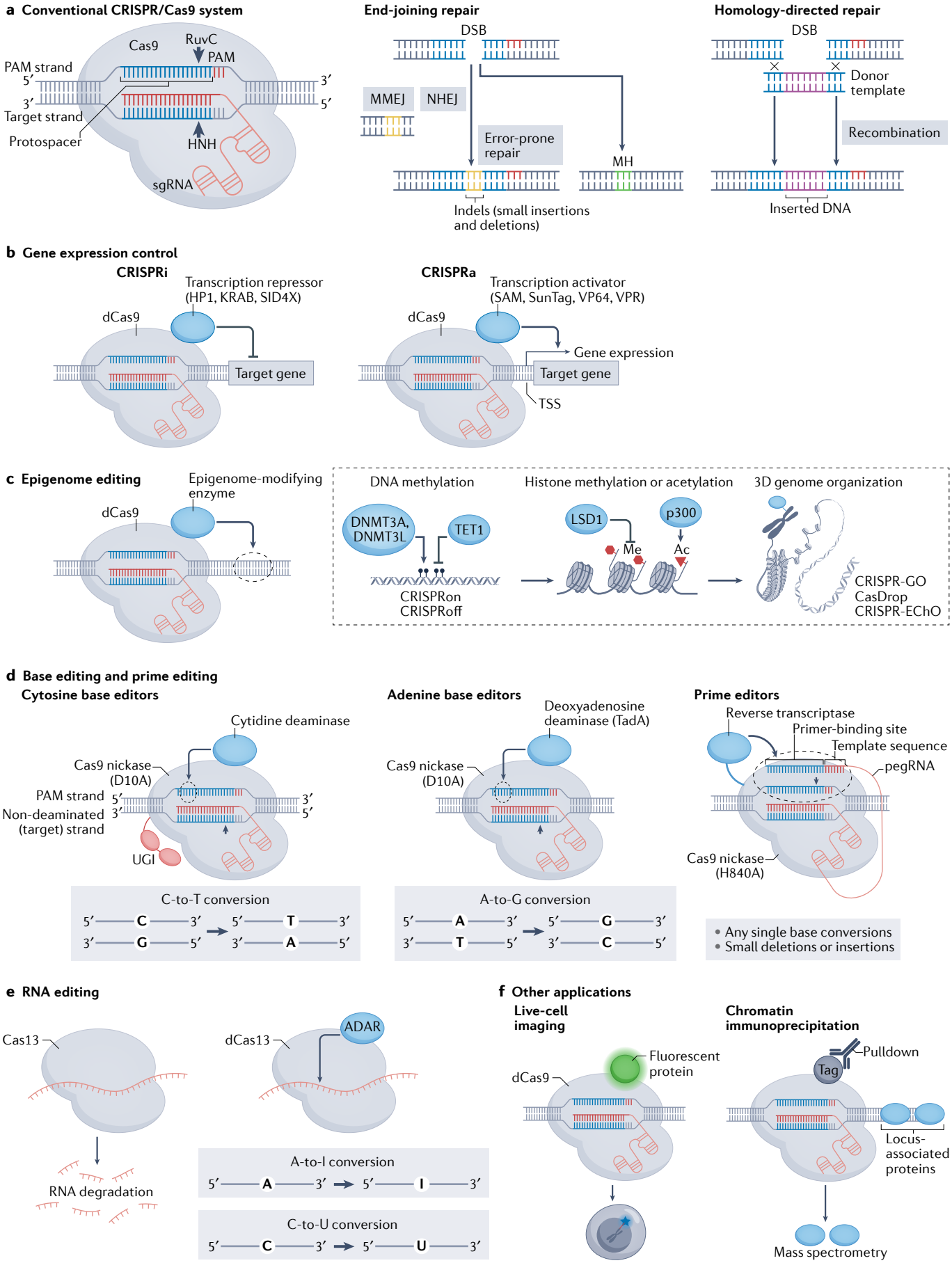
For transcriptional activation of endogenous genes, CRISPRa utilizes dCas9-based fusion proteins to recruit transcription activators to the target sites[4,11]. In mammalian cells, fusion proteins of dCas9 with VP64 or p65 activation domains were first shown to activate reporter genes or endogenous genes[22,30,31]. Approaches that use protein engineering or sgRNA engineering have enabled robust CRISPRa of endogenous genes without requiring multiple sgRNAs. The newest versions of CRISPRa systems include the SunTag array[32], VPR (a fusion protein of VP64, p65 and Rta)[33] and SAM (synergistic activation mediator)[34] (FIG. 2b). As with CRISPRi, CRISPRa tools are being used in various fields, including cardiovascular medicine[35–37] (TABLE 1).

Epigenome editing

Manipulation of epigenetic markers, such as DNA methylation, histone acetylation and histone methylation, can be achieved using dCas9 fusion proteins (FIG. 2c). DNA methylation is an epigenetic modification that causes gene silencing by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factors to DNA[38]. Therefore, the alteration of DNA methylation could be a target to control gene expression. In mammalian cells, DNA (cytosine-5)-methyltransferase 3A (DNMT3A) and DNMT3B catalyse methylation of unmethylated CpG sites, with DNMT3L as an important stimulatory cofactor. To introduce DNA methylation, dCas9 has been fused to the catalytic domain of DNMT3A, leading to site-specific methylation of the CpG islands around the target sites and repression of nearby genes[39–41]. By contrast, to remove DNA methylation, dCas9 has been fused to the catalytic domain of the demethylase TET1 (ten–eleven translocation methylcytosine dioxygenase 1), leading to the activation of endogenous genes[42,43]. In 2021, Nuñez et al.[44] and Nakamura et al.[45] reported on the permanent DNA methylation editors known as CRISPRon/CRISPRoff and dCas9–KAL, respectively. CRISPRoff and dCas9–KAL enabled gene silencing by introducing DNA methylation and repressive histone modifications with a single fusion protein of dCas9, DNMT3A, DNMT3L and KRAB[44,45]. Notably, transient induction of these two tools led to highly specific DNA methylation and gene repression, which were maintained even after cell division or differentiation of stem cells to neurons[44,45]. Furthermore, the epigenetic memories introduced by CRISPRoff can be reversed by CRISPRon (a fusion protein of dCas9, TET1 and XTEN80) by removing DNA methylation and recruiting transcriptional machinery[44].

Histone modifications have an important role in regulating chromatin structure and gene expression[46,47]. To control histone modifications, dCas9 has been fused to various histone-modifying enzymes. For example, dCas9 fused with the catalytic core of histone acetyltransferase p300 was shown to increase acetylation of histone

Induced pluripotent stem cells
(iPSCs). A type of stem cell that can be generated directly from somatic cells, such as fibroblasts or blood cells, by introducing specific sets of genes.


a Conventional CRISPR/Cas9 system
Cas9
RuvC
PAM
PAM strand
5′
3′
Target strand
Protospacer
HNH
sgRNA
3′
5′
End-joining repair
DSB
MMEJ
NHEJ
Error-prone repair
MH
Indels (small insertions and deletions)
Homology-directed repair
DSB
Donor template
Recombination
Inserted DNA
b Gene expression control
CRISPRi
Transcription repressor (HP1, KRAB, SID4X)
dCas9
Target gene
CRISPRa
Transcription activator (SAM, SunTag, VP64, VPR)
dCas9
Gene expression
Target gene
TSS
c Epigenome editing
Epigenome-modifying enzyme
dCas9
DNA methylation
DNMT3A, DNMT3L
TET1
CRISPRon
CRISPRoff
Histone methylation or acetylation
LSD1
p300
Me
Ac
3D genome organization
CRISPR-GO
CasDrop
CRISPR-EChO
d Base editing and prime editing
Cytosine base editors
Cytidine deaminase
Cas9 nickase (D10A)
PAM strand
Non-deaminated (target) strand
UGI
C-to-T conversion
5′ C 3′ 5′ T 3′
3′ G 5′ 3′ A 5′
Adenine base editors
Deoxyadenosine deaminase (TadA)
Cas9 nickase (D10A)
A-to-G conversion
5′ A 3′ 5′ G 3′
3′ T 5′ 3′ C 5′
Prime editors
Reverse transcriptase
Primer-binding site
Template sequence
pegRNA
Cas9 nickase (H840A)
• Any single base conversions
• Small deletions or insertions
e RNA editing
Cas13
RNA degradation
dCas13
ADAR
A-to-I conversion
5′ A 3′ 5′ I 3′
C-to-U conversion
5′ C 3′ 5′ U 3′
f Other applications
Live-cell imaging
dCas9
Fluorescent protein
Chromatin immunoprecipitation
Tag
Pulldown
Locus-associated proteins
Mass spectrometry

◀ Fig. 2 | **A wide variety of new CRISPR tools. a** | In the conventional CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system, the Cas9 nuclease is guided by a single guide RNA (sgRNA) and directed to the desired target sites. When Cas9 hybridizes to the target site, it generates double-strand DNA breaks (DSBs), which are made by two nuclease domains of Cas9: the RuvC domain (protospacer adjacent motif (PAM) strand) and the HNH domain (target strand). The DSBs are then repaired through endogenous pathways such as non-homologous end-joining (NHEJ), microhomology-mediated end-joining (MMEJ) and homology-directed repair. **b** | For gene-expression control, nuclease-deficient Cas9 (dCas9) is fused with transcriptional repressors or activators for CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa), respectively. **c** | To modify epigenetic markers, dCas9 is fused with epigenome-modifying enzymes such as DNA (cytosine-5)-methyltransferase 3A (DNMT3A; for DNA methylation), ten–eleven translocation methylcytosine dioxygenase 1 (TET1; for DNA unmethylation), lysine-specific histone demethylase 1 (LSD1; for histone unmethylation) or histone acetyltransferase p300 (p300; for histone acetylation). In addition, dCas9 can be used to modify 3D genome organization (CRISPR-GO, CasDrop and CRISPR-EChO). **d** | Base editors are typically a fusion protein combining a Cas nickase (such as Cas9 D10A) with a deaminase domain, which catalyses the substitution of a single nucleotide at the PAM strand in the R-loop. Prime editors consist of a Cas nickase (such as Cas9 H840A) fused with a reverse transcriptase domain. A prime editing guide RNA (pegRNA) contains a template of the desired sequence at the 3′ end as well as a target-specific spacer sequence. **e** | Cas13 family proteins bind to RNA instead of DNA. Cas13s can be used for knockdown of mRNAs. Fusion proteins of dCas13 coupled with an ADAR (adenosine deaminases acting on RNA) deaminase domain enable precise editing of mRNAs. **f** | dCas9 proteins fused with various effector proteins enable other applications, including live-cell imaging and chromatin immunoprecipitation. A, adenine; Ac, acetyl group; C, cytosine; G, guanine; I, inosine; KRAB, Krüppel-associated box; Me, methyl group; MH, microhomology; SAM, synergistic activation mediator; SID4X, four concatenated mSin3 interaction domains; T, thymine; TSS, transcription start site; U, uracil.

H3 lysine 27 (H3K27ac; a marker of active enhancers) and activate the target genes[48]. Similarly, dCas9 fused with lysine-specific histone demethylase 1 (LSD1) was shown to decrease the epigenetic modification levels of H3K4me2 and H3K27ac around the targeted enhancer region, leading to repression of the target genes[49]. Other examples of dCas9-based histone-modifying tools include histone methyltransferases (DOT1L, SMYD3 and PRDM9)[50,51] and histone deacetylases[52].

Several CRISPR tools can regulate larger genomic regions. For example, CRISPR-GO can control the spatial positioning of genomic loci in living cells[53]. CasDrop has been used to control liquid condensation at specific target loci to determine how chromatin is affected by liquid density[54]. CRISPR-EChO enables the tethering of heterochromatin components across tens of kilobases of endogenous genomic regions[55].

Base editing

Base editors have been developed to install targeted point mutations[19,56,57]. Although installing point mutations was possible with the conventional CRISPR/Cas9 system via HDR, this method had several limitations, including low efficiency, the requirement for donor DNA templates, the DNA repair response to DSBs and that the system cannot be used in postmitotic cells. By contrast, base editors allow the conversion of a single DNA base into another without requiring DSBs or donor templates. Current base editors consist of a catalytically impaired Cas nuclease (dCas9 or Cas9 nickase) fused to DNA deaminase enzymes[19]. Two classes of base editor have been developed: cytosine base editors (CBEs) and adenine base editors (ABEs) (FIG. 2d). CBEs and ABEs catalyse a C-to-T transition in the PAM strand (G-to-A transition in the target strand) and an A-to-G transition in the PAM strand (T-to-C transition in the target strand), respectively.

Mechanistically, when base editors bind to the target locus, hybridization of the sgRNAs to the target DNA strand initiates displacement of the PAM strand leading to the formation of a single-stranded DNA R-loop[19]. In the R-loop, PAM-distal nucleotides become accessible as single-stranded DNA to the deaminase domain of the base editors. In CBEs, cytidine deaminases convert cytosines within the R-loop to uracils, which are read as thymines by polymerases. In ABEs, deoxyadenosine deaminases (TadA) convert adenosines in the R-loop to inosines, which are read as guanines by polymerases. Most base editors, except the earliest versions, use a Cas nickase (such as Cas9 D10A) rather than a dCas protein because nicking the non-deaminated (target) strand can increase editing efficiency[19]. Additional improvements, such as adding uracil glycosylase inhibitor domains for CBEs, were made to increase editing efficiency and purity. The newest versions of base editors, such as BE3, BE4max and ABE8, have dramatically improved efficiency in mammalian cells, which allows unbiased parallel screening of genetic variants (CRISPR screening with base editors)[58,59] and in vivo base editing[12,13,60–69], as discussed below.

Although base editors do not directly generate DSBs, undesired editing can still occur either at the target sites or at off-target sites[19]. At the target sites, base editors can generate undesired by-products, including transversions (unintended conversions of nucleotides), bystander edits (unintended editing of C or A nucleotides in the editing window) and indel formation (potentially caused by base excision). At off-target sites, undesired DNA editing can occur in a Cas-dependent or Cas-independent manner[70–72]. Cas-dependent off-target editing is caused by the Cas domain of base editors and, therefore, these sites are shared between base editors and the corresponding Cas nucleases on which they are based. Cas-independent off-target editing is caused by the random deamination of nucleotides that are transiently accessible to the deaminase domain of base editors. In addition to unintended DNA editing, base editors have also been reported to introduce Cas-independent off-target RNA editing[73–75].

Prime editing

Prime editors are one of the newest types of genome-editing tool that can introduce all 12 possible types of point mutations (all six possible base-pair transitions and transversions), small insertions and small deletions without making DSBs or requiring DNA donor templates[19,76]. Prime editors are fusion proteins of a Cas nickase (such as Cas9 H840A) with an engineered reverse transcriptase domain (FIG. 2d). The guide RNAs that direct prime editors to the target sites, known as prime editing guide RNAs (pegRNAs), not only specify the target sites by the spacer sequences but also encode the desired edit in an extension at the 3′ end so that the reverse transcriptase domain can generate the edited DNA strand from the RNA-level template. Therefore, a single pegRNA performs the functions equivalent to

Table 1 | **Studies of new CRISPR tools for mechanistic cardiovascular research**

CRISPR tool	Cas nuclease	Species	Platform	Cells or tissues	Target genes	Delivery	Ref.
Repression	dCas9–KRAB	Human	In vitro	iPSC-CMs	*HERG*	Knock-in	23
	dCas9–KRAB	Human	In vitro	iPSC-CMs	*CALM2*	Lentiviral vector	24
	dCas9–KRAB	Zebrafish	In embryo	Vasculature	*tie1AS*	Microinjection	25
	dCas9–KRAB	Human	In vitro	HAECs	*PLPP3*	Transfection	26
	dCas9–KRAB	Zebrafish	In embryo	Endothelial cells	*tmem33*	Microinjection	27
	dCas9–KRAB	Human	In vitro	HAECs	*VEGFC*, *FGD6*, *KIF26B*	Transfection	28
Activation	dCas9–VPR	Mouse	In vitro	Fibroblast	*Gata4*, *Mef2C*, *Tbx5*, *Hand2*	Transfection	35
	dCas9–VPR	Mouse	In vivo	Heart	*Mef2d*, *Klf15*	Transgenic	36
	dCas9–VP64	Rat	In vitro	Cardiosphere-derived cells	*Gata4*, *Mef2c*, *Nkx2–5*, *Hand2*, *Tnnt2*	Lentiviral vector	37
Base editing (installation)	BE3	Mouse	In zygotes	Skeletal muscle	*Dmd*	Electroporation, microinjection	61
	BE3, ABE7.10	Rabbit	In zygotes	Systemic	*Mstn*, *Dmd*, *Tia1*, *Tyr*, *Lmna*	Microinjection	62
Prime editing	PE2	Mouse	In zygotes	Aorta, bladder, brain, heart	*Tspan2* promoter	Microinjection	77
Knockout screen	Cas9	Human	In vitro	hESC-CMs	NA	Lentiviral vector	120
	Cas9	Human	In vitro	iPSC-CMs	NA	Lentiviral vector	121
	Cas9	Zebrafish	In vivo	Heart	NA	Microfluidics	122
	Cas9	Mouse	In vivo	Heart	NA	Cas9: knock-in; sgRNA library: AAV vector	123

AAV, adeno-associated virus; dCas9, nuclease-deficient Cas9; HAEC, human aortic endothelial cell; hESC-CM, human embryonic stem cell-derived cardiomyocyte; iPSC-CM, induced pluripotent stem cell-derived cardiomyocyte; KRAB, Krüppel-associated box; NA, not available; sgRNA, single guide RNA.

both a sgRNA and a DNA template in the setting of HDR genome editing.

Currently, two nucleases are used for prime editing: PE1 and PE2 (REF.[76]). PE2 has an engineered reverse transcriptase domain with improved efficiency and so is preferable to PE1. After target binding, the RuvC nuclease domain of PE2 nicks the PAM strand. Next, depending on the desired sequence in the pegRNAs, the reverse transcriptase domain starts the synthesis of the edited DNA strand onto the 3′ end of the nicked DNA strand so that the newly synthesized DNA strand exists as a 3′ DNA flap. Endogenous DNA repair processes then allow the edited 3′ DNA flap to be incorporated into the editing site to replace the unedited strand with the desired sequence. Adding a simple sgRNA to direct PE2 to nick the unedited strand (a strategy known as PE3) increases the editing efficiency by stimulating the replacement of the unedited strand to the desired sequence[76]. In prime editing by PE2, Cas9-dependent off-target editing has been reported to be less frequent than that of conventional Cas9 at known Cas9 off-target sites[19,76,77], which could be related to the additional hybridization sequences in pegRNAs in addition to the spacer sequences[19]. Although prime editors have not yet been used intensively in cardiovascular research[77], they have strong potential especially for the characterization of genetic variants that cannot be introduced by base editors.

RNA editing

RNA editing has not been investigated as intensively as DNA editing, but four subtypes of Cas13 RNA-targeting enzymes have been reported to date: Cas13a, Cas13b, Cas13c and Cas13d[78–82] (FIG. 2e). Cas13 enzymes that are effective in human cells, and their catalytically inactive versions (dCas13s), have been used for diverse applications in mammalian cells, including transcript knockdown[80], live-cell transcript imaging[80] and RNA base editing[81]. For RNA editing, a fusion protein of dCas13 with an ADAR (adenosine deaminases acting on RNA) deaminase domain enables adenosine-to-inosine conversion[81] or cytidine-to-uridine conversion[82] at the RNA level.

RNA editing has several advantages over DNA editing[81,82]. First, because RNA editing occurs only at the RNA level without DNA damage, the effect is reversible and less cytotoxic. Therefore, RNA editing could be a safer option for therapeutic applications with fewer permanent off-target effects. Second, RNA editing does not rely on endogenous repair mechanisms such as NHEJ or HDR. RNA editing can therefore be used in most cell types, including postmitotic cells such as cardiomyocytes. Third, unlike Cas9 or Cas12a nucleases, Cas13 family proteins do not require a PAM sequence at the target sites. Therefore, the sequence of target genes or transcripts in RNA editing could be more flexible than that of DNA editing.

Other tools

dCas nucleases can be used as carriers to deliver effector proteins to target DNA (or RNA) sequences specified by sgRNAs. Therefore, many other applications of dCas nucleases are possible, including live-cell imaging and chromatin immunoprecipitation (ChIP) (FIG. 2f). dCas9 proteins fused with fluorescent markers have been used to visualize the genome in living cells[83,84]. Similarly, real-time RNA imaging can be achieved using dCas13 fusion proteins[85,86] without requiring genetic

Adeno-associated virus (AAV). A small, non-pathogenic virus that can infect many types of human cell and is often used as a vector for the delivery of gene therapy.

manipulation. dCas9 can also be used in combination with ChIP to pull down DNA-binding molecules that are physically interacting with a specific genomic locus[87,88].

Genome editing as a research tool

One of the primary applications of genome-editing technology is as a research tool to study biological mechanisms and disease pathophysiology. The applications of CRISPR as a research tool include the generation of genetic models, genetic (or epigenetic) perturbation to study the role of genes (or epigenetics), unbiased genetic screening, live-cell imaging and ChIP.

Genetic models

The CRISPR/Cas9 system has dramatically accelerated the generation of genetic models such as gene knockout or knock-in animal models (FIG. 3a). For example, a Cas9 protein and a sgRNA can be injected directly into single-cell mouse embryos to disrupt a gene, taking only weeks to yield knockout mice. Unlike conventional techniques, this method does not require the culturing of embryonic stem cells. Therefore, generating genetic models of other species, such as rats[89–92] and non-human primates[93,94], is also feasible. The new CRISPR tools described above provide more options for generating genetic models. In one study, mice with targeted point mutations in the genes encoding dystrophin (*Dmd*) or tyrosinase (*Tyr*) were generated by delivering BE3 mRNA or ribonucleoproteins into mouse zygotes via electroporation or microinjection[61]. Targeted point mutations were observed in 73% and 100% of blastocysts at the target site in *Dmd* and *Tyr*, respectively[61]. As shown in this study, base editors are useful in generating various animal models with single amino acid substitutions without requiring donor templates.

Instead of altering the genetic region of interest, the roles of multiple genes can be studied in a single model by generating stable expression models of new CRISPR tools and changing sgRNAs (FIG. 3b). Inducible CRISPRi human iPSC lines were generated by knocking in dCas9–KRAB to the AAVS1 (adeno-associated virus integration site 1) safe harbour locus to enable precise control of transcriptional silencing upon addition of doxycycline[23]. This platform was used to knock down endogenous genes in various cell types differentiated from iPSCs as well as undifferentiated iPSCs[23]. Knockdown of *HERG* (also known as *KCNH2*, which encodes the protein potassium voltage-gated channel subfamily H member 2) in iPSC-CMs resulted in a prolonged action potential duration, which recapitulated the phenotype observed in patients with LQTS[23]. Interestingly, compared with a knock-in line of the conventional CRISPR/Cas9 knockout system, CRISPRi knock-in lines enabled more homogeneous gene repression across cell populations[23]. In another study, two endogenous genes (*Mef2d* and *Klf15*) were activated in a mouse model with stable expression of dCas9–VPR under the control of a cardiac-specific promoter with sgRNAs delivered by adeno-associated virus (AAV) vectors (specifically, AAV9), resulting in hypertrophic phenotypes in the heart[36].

Table 2 | **Studies of new CRISPR tools for therapeutic genome editing in cardiovascular diseases**

Target disease	CRISPR tool(s)	Strategy	Cas nuclease	Species	Target tissues	Target gene	Delivery	Ref.
Hyperlipidaemia	Base editing	Disruption	BE3	Mouse	Liver	*Pcsk9*	Adenoviral vector	60
	Base editing	Disruption	ABEmax	Macaque	Liver	*PCSK9*	Lipid nanoparticle	13
	Base editing	Disruption	ABE8.8-m	Macaque	Liver	*PCSK9*	Lipid nanoparticle	12
	Repression	Knockdown	dSaCas9–KRAB	Mouse	Liver	*Pcsk9*	AAV vector	29
Duchenne muscular dystrophy	Base editing	Correction	ABE7.10	Mouse	Skeletal muscle	*Dmd*	AAV vector (embryo and adult)	63
	Base editing	Correction	iABE–NGA	Mouse	Skeletal muscle, heart	*Dmd*	AAV vector	64
	Base editing and prime editing	Exon skipping	ABEmax	Mouse	Skeletal muscle	*Dmd*	AAV vector	65
Marfan syndrome	Base editing	Correction	BE3	Human	Embryo	*FBN1*	Microinjection	145
Progeria (Hutchinson–Gilford syndrome)	Base editing	Correction	ABEmax–VRQR	Mouse	Liver, heart, aorta	*Lmna*	AAV vector	67
Hypertrophic cardiomyopathy	Base editing	Correction	ABEmax–NG	Mouse (in zygotes and in utero)	Heart	*Myh6*	Microinjection, AAV vector	68
Mucopolysaccharidosis	Base editing	Correction	ABEmax	Mouse	Liver, heart, brain	*Idua*	AAV vector	69
NA	Base editing	Installation and correction	BE3, ABEmax	Mouse	Heart, other tissues	*Dnmt1*	AAV vector	66

AAV, adeno-associated virus; NA, not available.

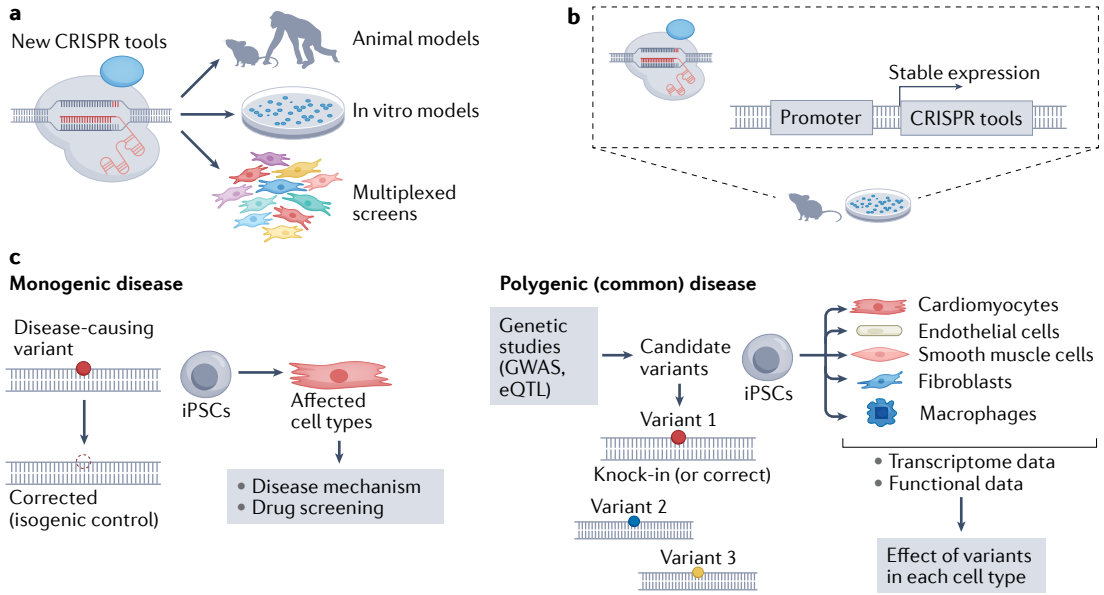


Fig. 3 | **New CRISPR technologies as research tools. a** | New CRISPR (clustered regularly interspaced short palindromic repeats) tools are used for genetic models and multiplexed screens. **b** | With stable expression models of diverse CRISPR tools, the roles of multiple genes, variants or epigenetic modifications can be studied in a single model by changing single guide RNAs. **c** | CRISPR genome editing in combination with induced pluripotent stem cell (iPSC) technology is useful for evaluating the effect of genetic variants. To study monogenic diseases, isogenic iPSCs are generated by correcting disease-causing mutations. To study variants involved in polygenic (common) diseases, candidate variants from genetic studies are introduced or corrected by genome editing to evaluate the effect of the variant. eQTL, expression quantitative trait locus; GWAS, genome-wide association study.

iPSCs and genome editing

The generation of isogenic cell lines from patient-derived iPSCs is another application of CRISPR as a research tool[95–97] (FIG. 3c). Human iPSCs are a promising platform to recapitulate disease phenotypes in vitro because they can mimic patient genetics and, in principle, be differentiated to any desired cell type[96–100]. However, wide variations between individual iPSC lines is often a challenge for the precise characterization of genetic variants[101]. First, each line of patient-derived iPSCs has a different genetic background. Second, the process of generating iPSCs from patients' somatic cells could affect epigenetics, pluripotency and capacity to differentiate. Therefore, simply comparing pathological iPSCs with healthy iPSCs might not be the ideal method of studying the effect of a particular genetic variant on disease phenotypes. A better strategy would be to generate corrected iPSC lines that differ only at the locus of interest from the original iPSC line, thereby eliminating complications caused by multiple variations[101]. Comparing original and corrected iPSCs enables precise characterization not only of monogenic variants that cause rare diseases but also of common variants that have a smaller effect size. For example, iPSC-CMs were generated from patients with rs2229774, a single-nucleotide polymorphism in *RARG* (encoding the retinoic acid receptor γ) that occurred in 15% of the population of the 1000 Genomes Project and which was identified in a genome-wide association study as a risk variant for cardiotoxicity induced by doxorubicin[102]. Using the CRISPR/Cas9 system to correct, knockout and overexpress *RARG*, the investigators demonstrated that iPSC-CMs from patients with the rs2229774 variant were more sensitive to doxorubicin and that RARG agonists could be protective against doxorubicin-induced cardiotoxicity[102]. To date, the conventional CRISPR/Cas9 system with HDR has often been used to correct or introduce genetic variants in iPSCs. Base editors and prime editors could be used in the future to accelerate the generation of isogenic iPSCs[65,70,103,104].

Other research applications

Other potential uses for CRISPR tools in research include the generation of reporter cell lines, live-cell imaging of genetic loci, pooled CRISPR screening and in vivo lineage tracing; these applications have been reviewed previously[4,11]. We summarize CRISPR screening in the section below because this method is becoming widely used and increasingly diverse with the emergence of new CRISPR tools.

CRISPR screening

Causative genes

Identifying causative genes or pathways in a phenotype or disease of interest is imperative for biological discovery and drug development. Progress in next-generation sequencing has enabled faster and easier genome-wide analysis of gene expression and epigenetic changes both in pooled cell populations and at the single-cell level. However, identifying causality is still a time-consuming process because most of the alterations in gene expression or epigenetics are caused by upstream changes or represent markers that are associated with the phenotype of interest. To address this issue, various approaches using multiplexed genetic perturbation screening have

Doxorubicin
A chemotherapy drug that is effective for many different types of cancer, including breast cancer and leukaemias, with a well-known adverse effect of cardiac toxicity.

allowed for the rapid identification of causative genes by knocking out many genes in parallel, either in a pooled or an arrayed manner, and then selecting cells with a phenotype of interest.

Until the emergence of CRISPR technology, pooled genetic screening was performed by chemical DNA mutagenesis or short hairpin RNA libraries, strategies that are limited by target size and poor efficiency. Owing to the simplicity of the CRISPR/Cas9 system, multiplexing many sgRNAs in a pooled library to perform efficient and accurate gene-knockout screening is now feasible, even on a genome-wide scale[105–108]. Furthermore, with the advent of new CRISPR tools, the types of genetic perturbation that can be examined via CRISPR screening are increasingly diverse and go beyond simple knockout to include inhibition[109], activation[107,110] and base editing[58,59].

Workflows

Pooled CRISPR screens require two main components: cells expressing Cas nucleases and a multiplexed sgRNA library (FIG. 4a). The cells used for CRISPR screens are typically immortalized cell lines with stable expression of Cas nucleases, either by viral delivery or targeted knock-in[106]. The sgRNA libraries for CRISPR screens are typically a pool of lentiviral plasmids that express different sgRNAs in each plasmid[106]. The sgRNAs in the pool are computationally designed for the candidate genes or all the genome-wide genes that could be targeted, and 5–10 sgRNAs per gene are usually designed. For example, genome-wide libraries targeting about 20,000 genes would contain 100,000–200,000 sgRNAs. An alternative method of delivering Cas nucleases and a sgRNA library to cells is to use all-in-one lentiviral libraries, in which each plasmid contains both a Cas nuclease and a sgRNA so that both components can be delivered simultaneously.

After Cas-expressing cells are infected with a lentiviral sgRNA library with an appropriate titre, each cell is labelled by a single sgRNA and the cells with a phenotype of interest are selected from the pooled cell population based on factors such as cell viability, drug resistance or fluorescent signals. The frequencies of cells expressing each sgRNA are then quantified to identify the sgRNAs that increase or diminish the phenotype of interest. As a result, the target genes of the screened sgRNAs are those that are causative (or repressive) for the phenotype of interest (FIG. 4a).

Readouts

In CRISPR screening, cells that show the phenotype of interest need to be collected to identify causative sgRNAs. Therefore, the readouts of pooled CRISPR screens need to be a phenotype that can be screened and which separate cells physically into two or more groups, the simplest often being cell growth or survival. For example, after the introduction of sgRNA libraries, cells are cultured with or without cytotoxic compounds, such as anticancer drugs, and the cells that survive are collected. By comparing sgRNA distribution in the surviving cells and untreated cells (or dead cells if possible), the sgRNAs that promote cellular survival or increase toxicity can be identified. Another readout that is commonly used is the fluorescence signal because cells can be separated on the basis of signal strength by fluorescence-activated cell sorting. The fluorescence signals can be labelled with antibodies, small molecules that monitor cellular activities, genetically encoded reporters or by the uptake of small particles (FIG. 4b).

Although the original protocols of CRISPR screening required physical separation of cells, as discussed above, technological developments have enabled single-cell RNA sequencing to be used as a high-dimensional readout in CRISPR screens. In single-cell CRISPR screens, such as Perturb-seq[111–113] and CROP-seq[114], the pooled cells labelled with various sgRNAs go through droplet-based single-cell capture instead of being separated into groups on the basis of phenotype, so that each droplet contains both mRNAs and the corresponding sgRNA from the same cell. The sgRNAs in individual droplets are identified during transcriptome sequencing. Consequently, each cell provides transcriptomic information under genetic perturbation by the corresponding sgRNA. Similarly, other types of single-cell sequencing, such as ATAC-seq (assay for transposase-accessible chromatin using sequencing) and multi-omics sequencing, have been used as a readout in CRISPR screens[115,116] (FIG. 4b).

Image-based screening that uses morphological phenotypes as a readout in CRISPR screens is also feasible. After the cells are labelled with sgRNAs, they are morphologically characterized by microscopy and the sgRNAs in individual cells are identified by in situ sequencing[117,118]. Although scalability is limited, this approach might be useful for analysing complex phenotypes such as contractile functions of cardiomyocytes.

Human iPSCs

CRISPR screens have typically been used in cancer cells to identify therapeutic targets to inhibit their proliferation or avoid resistance to anticancer drugs. However, CRISPR screens cannot easily be applied to human somatic cells, such as primary cardiomyocytes and neurons, owing to the high number of cells that need to be cultured. Typically, the number of cells that need to be cultured to avoid uncovered bias is >1,000-fold the number of sgRNAs in the library[105,106]. For example, typical genome-wide screens require >100 million cells per sample to be cultured. Therefore, using primary cells for CRISPR screening is not practical. Conversely, human iPSCs could be an ideal platform for CRISPR screening because iPSCs are expandable and can, in principle, be differentiated to any desired cell type. Genome-wide CRISPRi and CRISPRa screens were performed in iPSC-derived neurons to uncover pathways controlling the neuronal response to oxidative stress[119]. The researchers demonstrated that knockdown of the lysosomal protein prosaposin increased the response to oxidative stress exclusively in neurons by accelerating cellular ageing[119]. Similar approaches could be used in iPSC-CMs, endothelial cells or smooth muscle cells to study causative genes in cardiovascular diseases. With improved accuracy and efficiency, CRISPR screens in iPSCs could complement the information derived from genome-wide association studies (FIG. 4c).

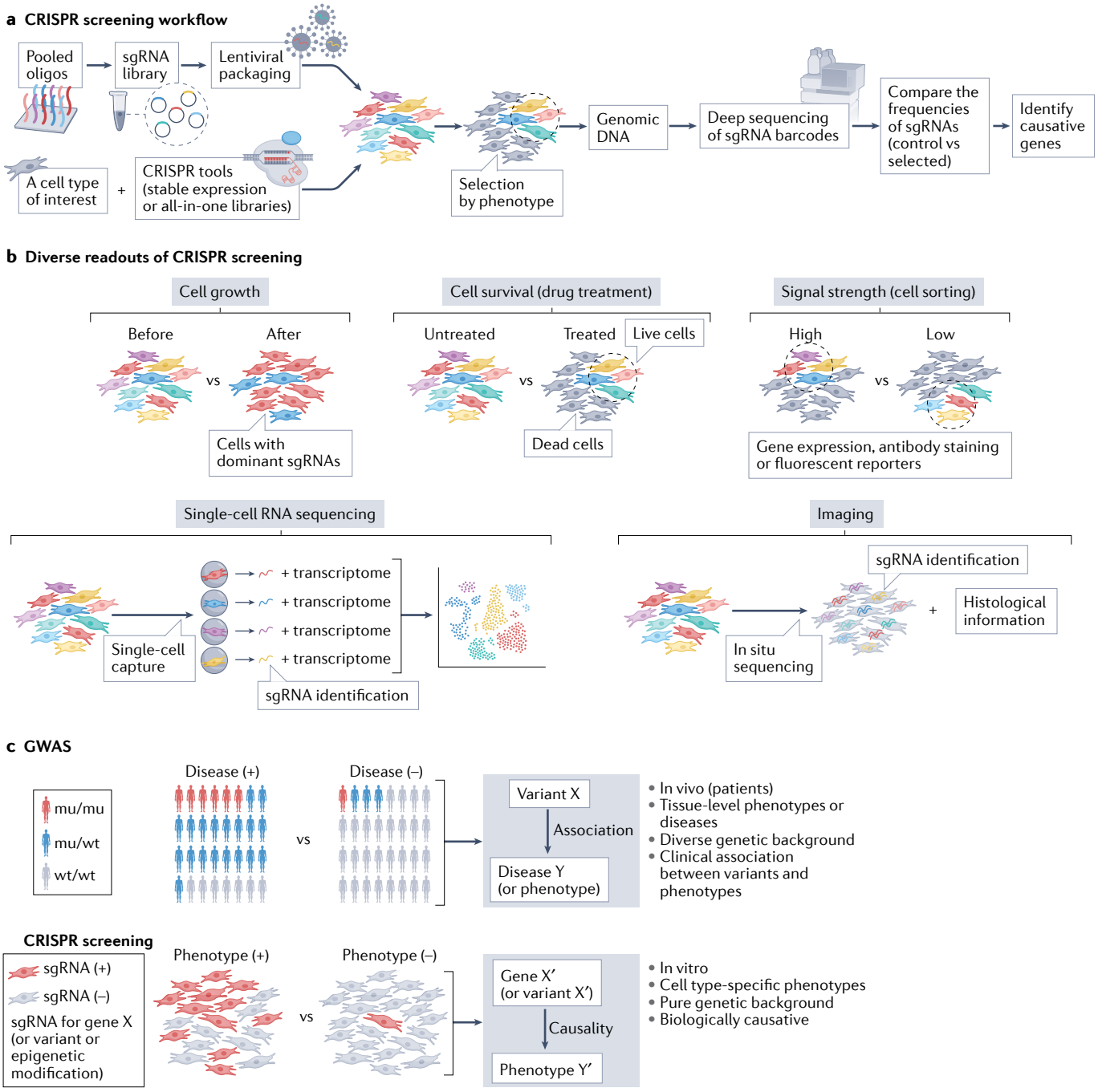


Fig. 4 | **Unbiased genetic screening with CRISPR tools. a** | Pooled CRISPR (clustered regularly interspaced short palindromic repeats) screens require two main components: a multiplexed single guide RNA (sgRNA) library and cells expressing Cas nucleases. The sgRNA libraries for CRISPR screens are typically a pool of lentiviral plasmids that express a different sgRNA in each plasmid. Cas-expressing cells are infected with a lentiviral sgRNA library, then each cell is labelled by a single sgRNA, which is integrated into the genome by a lentivirus. Next, the cells with a phenotype of interest are selected from the pooled cell population. The frequencies of cells expressing each sgRNA are then quantified by next-generation sequencing to identify the sgRNAs that increase or diminish the phenotype of interest. **b** | The readouts that can be used as a phenotype in CRISPR screens are cell growth, survival and signal strength. Alternatively, single-cell RNA sequencing could be used as a high-dimensional readout. Each droplet contains both mRNAs and the corresponding sgRNA from the same cell; therefore, each cell provides transcriptomic information under a particular genetic perturbation (Perturb-seq or CROP-seq). Moreover, image-based phenotypes can also be used as a readout. The cells are morphologically analysed by microscopy, and the sgRNAs in individual cells are identified by in situ sequencing. **c** | In case–control genome-wide association studies (GWAS), association tests for >1 million variants are performed to determine whether allele frequency is significantly altered between a group with the disease of interest and a healthy group. Similarly, CRISPR screens can be used to compare the frequencies of individual sgRNAs between a phenotype-positive group and a phenotype-negative group. With the new CRISPR tools, such as base editors or prime editors, generating a pooled cell population in which each cell carries a different variant is also feasible. CRISPR screens could therefore complement the information from GWAS. mu, mutation carrier; wt, wild type.

Cardiovascular research

In cardiovascular research, CRISPR screens have primarily been used in cardiac development and maturation[120–123]. For example, genome-wide CRISPR knockout screens were performed in human embryonic stem cells and identified *ZIC2* (encoding Zic family member 2) as a key regulator of cardiac progenitor formation[120]. In vivo CRISPR screens have been also reported. A platform known as MIC-Drop (multiplexed intermixed CRISPR droplets) has been developed that combines droplet microfluidics, single-needle en masse CRISPR ribonucleoprotein injections and DNA barcoding to enable large-scale functional genetic screens in zebrafish[122]. In a MIC-Drop screen of 188 poorly characterized genes, the investigators discovered several genes important for cardiac development and function, including *GSTM3* (encoding glutathione S-transferase mu 3)[122], a variant of which has been identified as a risk factor for Brugada syndrome[124]. In another study, in vivo CRISPR screens were performed in a mouse model to identify key regulators of cardiomyocyte maturation[123,125]. The researchers delivered a sgRNA library, containing approximately 15,000 sgRNAs, with AAV vectors to neonate Cas9-expressing mice. At 4 weeks, cardiomyocytes were sorted on the basis of MYH7 expression, a marker of cell immaturity. *Rnf20* and *Rnf40* (encoding ring finger protein 20 and ring finger protein 40, respectively) were identified as key epigenetic regulators of cardiac maturation[123,125].

Therapeutic genome editing

The development of CRISPR technologies has increased the potential to treat disease with therapeutic genome editing, which removes or corrects harmful mutations or introduces protective modifications to the patient's genome[126,127]. New CRISPR tools, such as base editors, have advanced therapeutic genome editing by improving efficiency and reducing potential adverse effects.

Many inherited or de novo monogenetic cardiovascular diseases (for example, dilated cardiomyopathy, familial pulmonary hypertension, hypertrophic cardiomyopathy, LQTS, Marfan syndrome and muscular dystrophies) could theoretically be cured by editing disease-causing mutations in cardiomyocytes or vascular cells, as shown in iPSC disease-modelling studies (FIG. 4a). At present, however, editing the genome of the heart and vessels is challenging owing to the low editing efficiency in somatic cells and the lack of efficient delivery methods. Studies of therapeutic genome editing in cardiovascular medicine have been restricted to the liver, particularly those targeting *PCSK9* for hyperlipidaemia. In the remainder of this Review, we discuss advances and challenges in therapeutic genome editing with new CRISPR tools in the field of cardiovascular medicine (TABLE 2).

Target diseases

Target diseases for therapeutic genome editing can be divided into two categories: monogenic diseases and polygenic diseases. For monogenic diseases, such as Duchenne muscular dystrophy (DMD), that are caused by pathogenic single mutations, the target loci are the causative genomic regions in the affected cell types (or tissues). By contrast, treatment or prevention of polygenic diseases, including common conditions such as hyperlipidaemia, could be achieved by editing non-causal genes to introduce beneficial variants or protective modifications. For example, *PCSK9* and *CCR5* (encoding C-C motif chemokine receptor 5) could be targets for treating hyperlipidaemia and HIV infection, respectively, even if patients do not have causal mutations in these genes.

Strategies

Genome-editing strategies for the prevention or treatment of disease involve either the disruption or correction of target genes (FIG. 5a).

Gene disruption

The most straightforward strategy is to disrupt a gene or region that is harmful or the disruption of which is protective such as disrupting *PCSK9* to reduce blood LDL-cholesterol levels. PCSK9 is a serine protease secreted mainly from the liver that binds to the LDL receptor and promotes the endocytosis and lysosomal degradation of the receptor, leading to reduced uptake of LDL-cholesterol from the blood. Rare gain-of-function mutations in *PCSK9* are known to cause familial hypercholesterolaemia[128]. By contrast, loss-of-function variants in *PCSK9* that occur in 2–3% of particular ethnic populations are associated with reduced plasma LDL-cholesterol levels and substantial protection against coronary heart disease, without causing adverse phenotypes[129]. Therefore, therapies targeting *PCSK9* could be beneficial in patients treated with statins who continue to have persistently high LDL-cholesterol levels. Monoclonal antibodies against PCSK9 (such as alirocumab and evolocumab) have already been used in clinics. Whereas monoclonal antibodies require periodic administration, genome editing of *PCSK9* could be a one-time therapy to achieve permanent knockout.

Murine studies have shown that the conventional CRISPR/Cas9 system with NHEJ genome editing can achieve permanent disruption of *Pcsk9* in the liver, resulting in substantially reduced plasma levels of PCSK9 and LDL-cholesterol[130–132]. The use of base editors to knock out *Pcsk9* by introducing nonsense or splice-site mutations has also been reported. In vivo base editing of *Pcsk9* in mice using a cytosine base editor, BE3, delivered with a sgRNA in an adenoviral vector, introduced a nonsense mutation in *Pcsk9* in the liver, leading to >50% reduction in blood PCSK9 levels and a 30% reduction in blood LDL-cholesterol levels[60]. Two studies have described the use of adenine base editors to disrupt *PCSK9* in cynomolgus macaques (*Macaca fascicularis*). The adenine base editor ABEmax was used to knock out *PCSK9* by introducing a splice-site mutation[13]. An mRNA encoding ABEmax together with a chemically modified sgRNA was formulated in lipid nanoparticles and injected intravenously. Among the four groups tested (low dose or high dose, as either a single dose or two doses with a 2-week interval between dosing), the two high-dose treatments resulted in ~30% editing at the DNA level, a 40% reduction in serum PCSK9 level

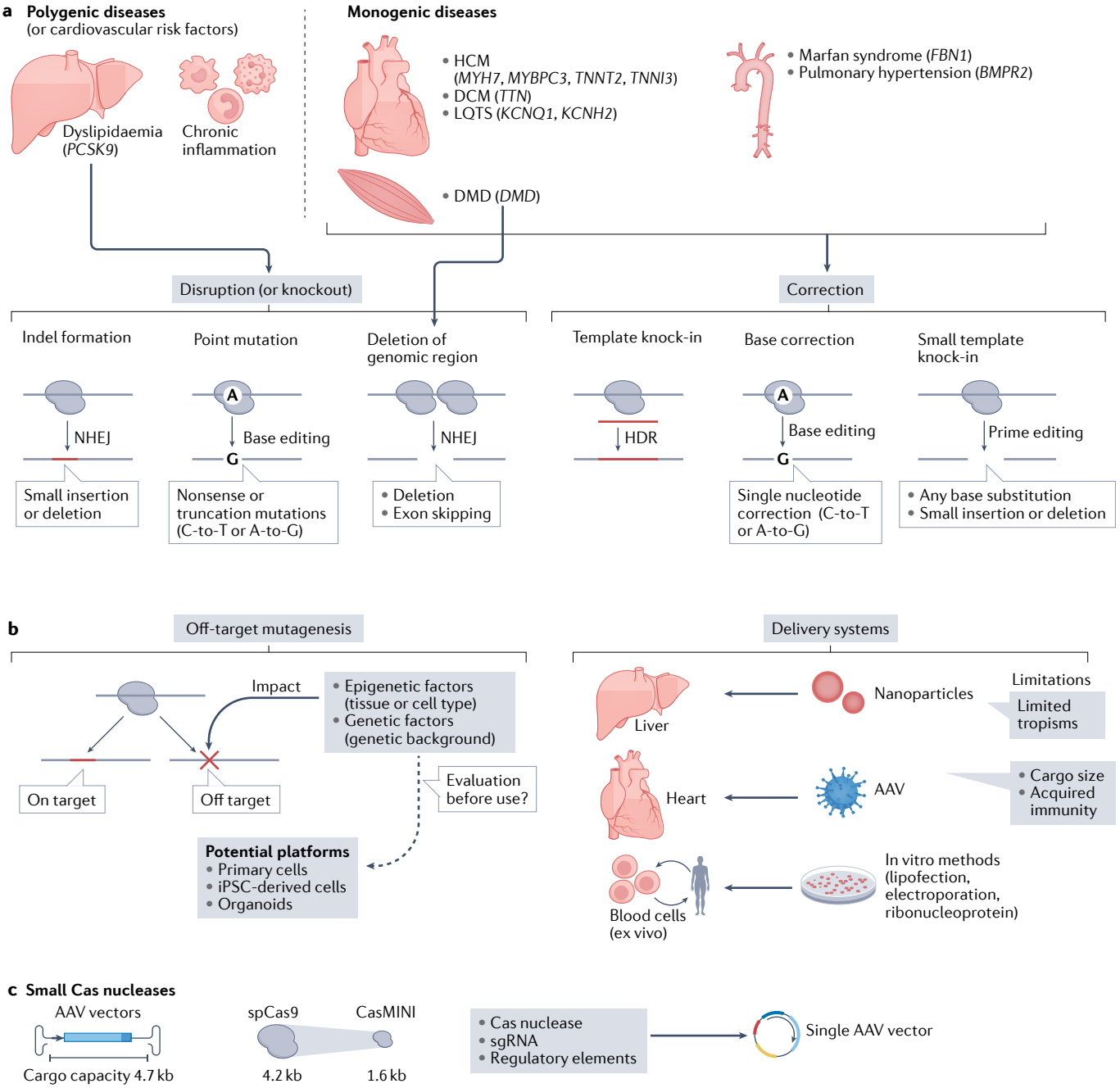


Fig. 5 | **Therapeutic genome editing in cardiovascular disease. a** | Many inherited or de novo monogenetic, cardiovascular disorders could theoretically be cured by correcting mutations. By contrast, to treat or prevent polygenic diseases (such as hyperlipidaemia), which are more common than monogenic diseases, non-causal genes are edited to introduce beneficial variants or protective modifications. **b** | Current challenges in therapeutic genome editing include the lack of a method to evaluate off-target editing (mutagenesis) and the lack of a system to deliver genome editors efficiently and specifically to target tissues. Off-target mutagenesis would vary between different tissues or cell types (owing to epigenetic differences) and between individuals (owing to genetic background). To minimize undesired off-target editing, pretreatment evaluation using patient-derived cells would be necessary. Nanoparticles are currently the most efficient method to deliver CRISPR (clustered regularly interspaced short palindromic repeats) tools to the liver, but the tropism is limited to the liver or spleen. Delivery systems using adeno-associated viruses (AAV) have broader tropisms, including to the heart. The disadvantages of AAV vectors are their limited cargo capacity and them being affected by the immune response. **c** | To address the cargo-size limitation of AAV vectors (about 4.7 kb), smaller Cas nucleases have been developed so that the coding sequence of Cas nucleases, single guide RNA (sgRNAs) and regulatory elements can be packed together in a single AAV vector. A, adenine; C, cytosine; DCM, dilated cardiomyopathy; DMD, Duchenne muscular dystrophy; G, guanine; HCM, hypertrophic cardiomyopathy; HDR, homology-directed repair; iPSC, induced pluripotent stem cell; LQTS, long QT syndrome; NHEJ, non-homologous end-joining; spCas9, *Streptococcus pyogenes* Cas9; T, thymine.

and a 20% reduction in serum LDL-cholesterol level[13]. In another study, ABE8.8, one of the latest adenine base editors[133], was used to knockout *PCSK9* by introducing the same splice-site mutation[12]. A single intravenous infusion of lipid nanoparticles containing ABE8.8 mRNA and a chemically modified sgRNA resulted in

surprisingly high editing efficiency: 66% editing at the DNA level, a 90% reduction in blood PCSK9 level and a 60% reduction in blood LDL-cholesterol level[12]. In both studies, almost no detectable off-target mutagenesis and minimal on-target editing in other tissues were observed. Although one study showed greater editing efficiency than the other, which could be related to the different versions of ABE and dosing schedules (the effect of repeated dosing might have been diminished by immune responses), these studies on non-human primates clearly demonstrate the powerful potential of base editors as an efficient and safe tool for therapeutic gene knockout.

Successful gene disruption in the human liver has been reported in a study targeting transthyretin amyloidosis (also known as ATTR amyloidosis)[134]. Transthyretin amyloidosis is a progressive, fatal disease caused by the accumulation of amyloid fibrils composed of misfolded transthyretin (TTR) protein in the nerves and heart, leading to amyloid polyneuropathy, cardiomyopathy or both. Hereditary forms of transthyretin amyloidosis can be caused by pathogenic mutations in *TTR*. In a clinical study, six patients with hereditary transthyretin amyloidosis with polyneuropathy received in vivo gene-editing therapy with NTLA-2001, which disrupts TTR in hepatocytes[134]. NTLA-2001 contains a sgRNA targeting human TTR and mRNA of *S. pyogenes* Cas9 in a lipid nanoparticle delivery system with liver tropism. According to preclinical evaluations, the editing efficiency at the DNA level was >90% and >70% in human primary hepatocytes and cynomolgus macaque livers, respectively[131]. All seven loci identified as possible off-target editing sites in the human genome were in non-coding regions, and no evidence of editing at these loci was identified in human primary hepatocytes. In the clinical trial, patients received a single intravenous infusion of either 0.1 mg/kg or 0.3 mg/kg of NTLA-2001. Few adverse events, all of which were mild (grade 1), were observed. The mean reduction in serum TTR protein concentration at day 28 was 52% in the low-dose group and 87% in the high-dose group[134]. Although this clinical trial is ongoing and long-term outcomes are unknown, the favourable early results provide powerful proof of concept for in vivo therapeutic genome editing in humans.

Another example of the disruption strategy is 'exon skipping' for the treatment of DMD, a recessive, muscle-wasting disorder linked to the X chromosome. DMD is caused by pathogenic mutations in *DMD*, which encodes the large cytoskeletal protein dystrophin, and is one of the most prevalent fatal genetic diseases in young boys[135]. These mutations are typically deletions of one or more exons, resulting in abnormal reading frames and a complete loss of functional dystrophin protein. Patients are usually diagnosed in childhood, have abnormal cardiac function from their early teens, and die in their twenties from heart or respiratory failure. Exon skipping aims to restore the reading frame by skipping the affected exons, which could recover the function of dystrophin. For the most common type of DMD, with mutations in exon 51 or 53, oligonucleotide-mediated drugs that the effect exon skipping are already approved for clinical use (eteplirsen, golodirsen and viltolarsen)[136]. Therefore, exon skipping by genome editing could permanently rescue the function of dystrophin. In animal models of DMD, the conventional CRISPR/Cas9 system with two sgRNAs flanking the target exon has been tested to remove the affected exon via NHEJ editing[137–144]. The efficacy of this strategy was confirmed in a dog model with a naturally occurring mutation that shows the clinical phenotypes of human DMD[142]. The investigators demonstrated a substantial increase in dystrophin protein levels in skeletal and heart muscles after the introduction of exon skipping[142].

Gene correction. The second genome-editing strategy is gene correction, which aims to restore the sequence and function of mutated genes and is therefore more challenging than gene disruption. With the conventional CRISPR/Cas9 system, gene correction can be achieved via HDR in ex vivo or in vitro platforms, in which successfully edited cells can be purified and amplified. However, this approach has low editing efficiency and requires cell selection, which cannot be achieved in vivo. Moreover, HDR is not active in non-dividing cells such as cardiomyocytes. Therefore, this technique cannot be applied to in vivo gene correction for cardiac diseases.

With the advent of new CRISPR tools that do not rely on HDR, particularly base editors, in vivo gene correction is becoming more feasible. Base editors that have been developed so far can achieve precise editing of C-to-T or A-to-G substitution without making DSBs, limiting the cellular DNA damage response. An estimated 30% of genetic variants in the ClinVar database are transition point mutations (C→T, G→A, A→G or T→C), which could be corrected (or introduced) either by C-to-T substitution or A-to-G substitution[19,20,76]. As the efficiency and specificity of base editors are progressively improved, in vivo gene correction is being attempted in animal models. For example, the treatment of DMD could shift from exon skipping to precise correction of the point mutations with base editors. A nonsense mutation in *Dmd* was successfully corrected in mouse skeletal muscles in vivo with the use of ABEs delivered by two AAV vectors[63]. In another study, a modified ABE (iABE–NGA) was used in *mdx*^{4cv} mice to correct a point mutation with a premature stop codon (CAA-to-TAA) in exon 53 of *Dmd*, resulting in restoration of dystrophin and functional improvement[64]. Of note, editing efficiency in the heart at 10 months in this study was >80% at the RNA level and >95% at the protein level[64]. Successful editing of a hypertrophic cardiomyopathy-associated pathogenic mutation in *Myh6* (R404Q) in zygotes and in utero using an ABE has also been reported[68]. These findings suggest that base editors have the potential to cure genetic cardiac diseases, such as hypertrophic cardiomyopathy, dilated cardiomyopathy and LQTS, if the pathogenic variants are T-to-C or G-to-A transitions (A-to-G or C-to-T on the complementary strand). Similarly, vascular diseases could be a target for gene correction therapy. BE3 was used to correct mutated *FBN1* in human embryos as a potential strategy to prevent Marfan syndrome[145].

Therapeutic CRISPR without DNA editing

Other CRISPR tools that do not alter genomic DNA sequences also have potential therapeutic applications. Theoretically, the CRISPRi system could be applied to diseases that are currently targeted by monoclonal antibodies. CRISPRa could be even more attractive than CRISPRi because gene activation is difficult to achieve with currently available molecularly targeted drugs. RNA editors could provide a therapeutic effect similar to that of base editors, without changing DNA sequences or causing a DNA damage response. To date, very few studies have been performed on the therapeutic use of these non-genome-editing CRISPR tools. A CRISPRi system was used to knock down *Pcsk9* in mice[29]. Systemic administration of AAV vectors expressing dSaCas9–KRAB and a *Pcsk9*-targeting sgRNA resulted in significant reductions in the serum levels of PCSK9 and LDL-cholesterol.

Current challenges

Safety is the most crucial challenge for genome-editing therapy in humans[126,127]. In addition to the adverse effects shared with other gene therapies, such as toxicity of drug-delivery reagents and the immune response, therapeutic genome editing also carries the risk of off-target mutagenesis, which generates de novo mutations at undesired genetic loci. Although the new CRISPR tools that do not make DSBs theoretically reduce off-target editing, base editors have been reported to cause Cas-independent off-target changes that are distinct from those of the conventional Cas nucleases[70–72]. Base editors could also cause off-target editing at the RNA level[73–75]. Off-target editing is still an important issue because it could lead to undesired, possibly permanent, phenotypes. In the heart, off-target mutagenesis can cause fatal arrhythmic problems even if the mutations occur in only a small percentage of cardiac cells, making genome editing for cardiac diseases particularly challenging.

Currently, there is no established way to predict off-target mutations before the use of genome editing in patients. Unlike model organisms, such as mice and rats, each patient has a different genetic background and the sites of potential off-target editing will differ between individuals. Off-target editing also depends on the tissue or cell type because epigenetic status affects the accessibility of genome editors to chromatin. Moreover, the off-target sites of new CRISPR tools could differ from those of well-characterized *S. pyogenes* Cas9 (REFS[70–72]). Pretreatment evaluations, such as in silico analysis, in vitro cleavage of the patient genome or in vitro surrogate systems using patient-derived cells, should be performed for each patient to minimize the risk of off-target mutations. In one of the studies of therapeutic base editing of *PCSK9* in monkeys discussed earlier, in vitro off-target editing in primary hepatocytes showed a concordant result with in vivo off-target editing detected in liver biopsy samples[12]. This finding suggests that in vitro culture of primary cells is an appropriate surrogate to evaluate off-target editing in vivo. On this basis, human primary hepatocytes from four individuals were analysed to identify potential off-target sites[12]. No potential off-target sites were detected in approximately 70 candidate sites in the human genome[12]. For cell types that are not suitable for primary culture, such as cardiomyocytes, iPSC-derived cells from patients could be useful for this purpose (FIG. 5b).

Another challenge in therapeutic genome editing is the lack of specific and efficient delivery methods. Viral vectors, particularly AAV vectors, are currently the only means of delivering genome editors to the heart. Eleven serotypes occur naturally, and there are >100 AAV variants with different amino acid sequences in the capsid. Each serotype or variant has a tropism for a type of tissue such as eye, brain, liver or muscle[146]. For cardiac gene therapy, AAV vectors have been used in phase II clinical trials to deliver *SERCA2A* (also known as *ATP2A2*; encoding sarcoplasmic reticulum Ca^{2+} ATPase 2a) to the heart[147,148]. One of the limitations of AAV vectors is their packaging capacity (up to 4.7 kb). As the coding sequence of *S. pyogenes* Cas9 is 4.2 kb, and most other Cas nucleases are a similar size, little space remains to package sgRNAs or control elements. One way to overcome this limitation is to divide Cas nucleases into two AAV vectors[63,64,149]. Efforts to discover or engineer smaller Cas nucleases (such as NmeCas9, CjCas9, Cas12b, CasX and Cas13bt) that are suitable for packaging in AAV vectors are under way[150–155]. Of note, the development of compact and effective Cas effectors, such as the miniature CasMINI system (~1.6 kb)[156], offers the potential to fit all molecular components into a single AAV vector and improve delivery efficiency (FIG. 5c).

The patient's immune response is another limitation to the use of AAV vectors. They can be delivered only once because patients acquire immunity to the serotype after the first administration[146]. Some patients might already be immune to the AAV serotypes or variants before treatment because of natural exposure to similar viruses[146] (FIG. 5b). Of note, pre-existing adaptive immune responses to Cas nucleases, which are not limited to viral vectors, also need to be considered[157].

Nanoparticles also have the potential to deliver genome editors to the target tissues[158]. In the two studies of *PCSK9* knockout in monkeys discussed earlier[12,13], lipid nanoparticles were used to deliver ABEs to the liver. One advantage of nanoparticles over AAV vectors is their transient delivery because AAV vectors can cause permanent integration or long-lasting expression, leading to greater off-target effects and stronger immune responses[158]. However, nanoparticles have limited biodistribution, tending to accumulate in the liver and spleen[158], and so targeting other tissues, such as the heart, is difficult. Moreover, cardiac-specific delivery by nanoparticles is also challenging because cardiomyocytes lack unique cell-surface markers. In summary, developing viral vectors with increased cargo capacity or nanoparticles with tropisms for diverse tissues would be an important step towards successful therapeutic genome editing for cardiovascular diseases.

Conclusions

With the growing number of CRISPR tools being developed, the functions and applications of these tools have diversified and now range from gene-expression control to epigenome editing, RNA editing and base

editing. These powerful new tools are being leveraged to study mechanisms of disease and hold therapeutic promise for treating common conditions such as hyperlipidaemia. Among the research applications of CRISPR tools, unbiased multiplexed perturbation or screens to identify causative genes show potential for discovering biological mechanisms and novel drug targets. The therapeutic applications for CRISPR tools are increasing in parallel with improvements in efficiency and specificity of in vivo genome editing. Challenges to the clinical application of these technologies include the inability to predict off-target mutagenesis and inefficient delivery methods, particularly for the tissues of the heart and vessels.

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