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Gene editing in common cardiovascular diseases

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ABSTRACT

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide, highlighting the high socioeconomic impact. Current treatment strategies like compound-based drugs or surgeries are often limited. On the one hand, systemic administration of substances is frequently associated with adverse side effects; on the other hand, they typically provide only short-time effects requiring daily intake. Thus, new therapeutic approaches and concepts are urgently needed. The advent of CRISPR-Cas9 genome editing offers great promise for the correction of disease-causing hereditary mutations. As such mutations are often very rare, gene editing strategies to correct them are not broadly applicable to many patients. Notably, there is recent evidence that gene editing technology can also be deployed to disrupt common pathogenic signaling cascades in a targeted, specific, and efficient manner, which offers a more generalizable approach. However, several challenges remain to be addressed ranging from the optimization of the editing strategy itself to a suitable delivery strategy up to potential immune responses to the editing components. This review article discusses important CRISPR-Cas9-based gene editing approaches with their advantages and drawbacks and outlines opportunities in their application for treatment of cardiovascular diseases.

Contents

Abbreviations: AAV, adeno-associated virus; ABE, adenine base editor; AuNP, gold nanoparticle; AYBE, adenine transversion base editor; CaMKIIδ, Calcium/calmodulin-dependent protein kinase IIδ; Cas, CRISPR-associated protein; CBE, cytosine base editor; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, CRISPR interference; crRNA, CRISPR RNA; dCas9, dead Cas9; DMD, Duchenne muscular dystrophy; DSB, double-strand break; GBE, glycosylase base editor; HCM, hypertrophic cardiomyopathy; INDEL, insertion or deletion; LNP, lipid nanoparticle; nCas9, nickase Cas9; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; PE, prime editor; sgRNA, single-guide RNA; spCas9, Cas9 derived from *Streptococcus pyogenes*; tracrRNA, transactivating CRISPR RNA; UGI, uracil glycosylase inhibitors; UNG, uracil DNA N-glycosylase.

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1. Introduction

Cardiovascular diseases are the most common cause of morbidity and mortality worldwide and the frequency is expected to increase in the coming years (Collaborators GBDRF, 2020; Virani et al., 2021). Even though there have been recent advances in heart disease therapy (e.g., with gliflozins), patients' prognosis is still dramatically impaired as treatments are often not fully effective or accompanied by severe adverse side effects (Heidenreich et al., 2022). The latter can be caused by non-specific binding of a compound to an off-target protein, but also by on-target binding in another organ, where the effector protein is not necessarily pathogenic. For instance, ATP-competitive inhibitors have been shown to block also several off-target enzymes (Nassal et al., 2020; Pellicena & Schulman, 2014). Additionally, conventional compound-based strategies rely on patients' compliance to take their prescribed medications. Studies show that up to 40 % of all patients do not adhere to their prescribed medications when three or more pills are prescribed (Anderson & Funnell, 2000; Gupta et al., 2017; Kulkarni et al., 2006). These challenges and limitations are observed in many different therapeutic approaches, and often prevents them from being translated into the clinical. One prominent example in the cardiovascular field is the development of inhibitors of Calcium/calmodulin-dependent protein kinase IIδ (CaMKIIδ). CaMKIIδ has been implicated in various forms of cardiac pathology, including myocardial infarction, heart failure, arrhythmias, and sleep-disordered breathing. Even though there is a multitude of studies showing benefits of CaMKIIδ inhibition in preclinical models, there is no such drug available for patients due to the above-discussed challenges and limitations of traditional compound-based strategies (Backs et al., 2009; Bers, 2014; Gray et al., 2017; Lebek et al., 2020; Lebek, Chemello, et al., 2023; Little et al., 2009; Mattiazzi et al., 2015; Pogwizd et al., 2001; Ryan et al., 2022; Singh & Anderson, 2011; Watkins et al., 2011). Thus, developing new and optimized therapeutic approaches requires a detailed understanding of the pathomechanisms as well as advanced biotechnological tools. Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) gene editing has emerged as one of the most promising biotechnological tools in our time. Careful design of an optimized editing strategy and utilization of a tissue-specific promoter to drive the expression of the editing components substantially increase target- and organ-specificity, respectively, which decreases the risk of potential adverse side (Anzalone et al., 2020; Lebek et al., 2024; Lebek, Chemello, et al., 2023). Plus, this approach can induce a permanent edit in the genome, thereby bypassing the requirement of daily drug intake (Karri et al., 2022). The aim of this article is to review the currently available genome editing tools, highlighting advantages and potential applications, as well as discussing different delivery strategies. Particular focus is on the usage of CRISPR-Cas9 technology for the correction of hereditary cardiovascular diseases and the disruption of common pathogenic signaling cascades.

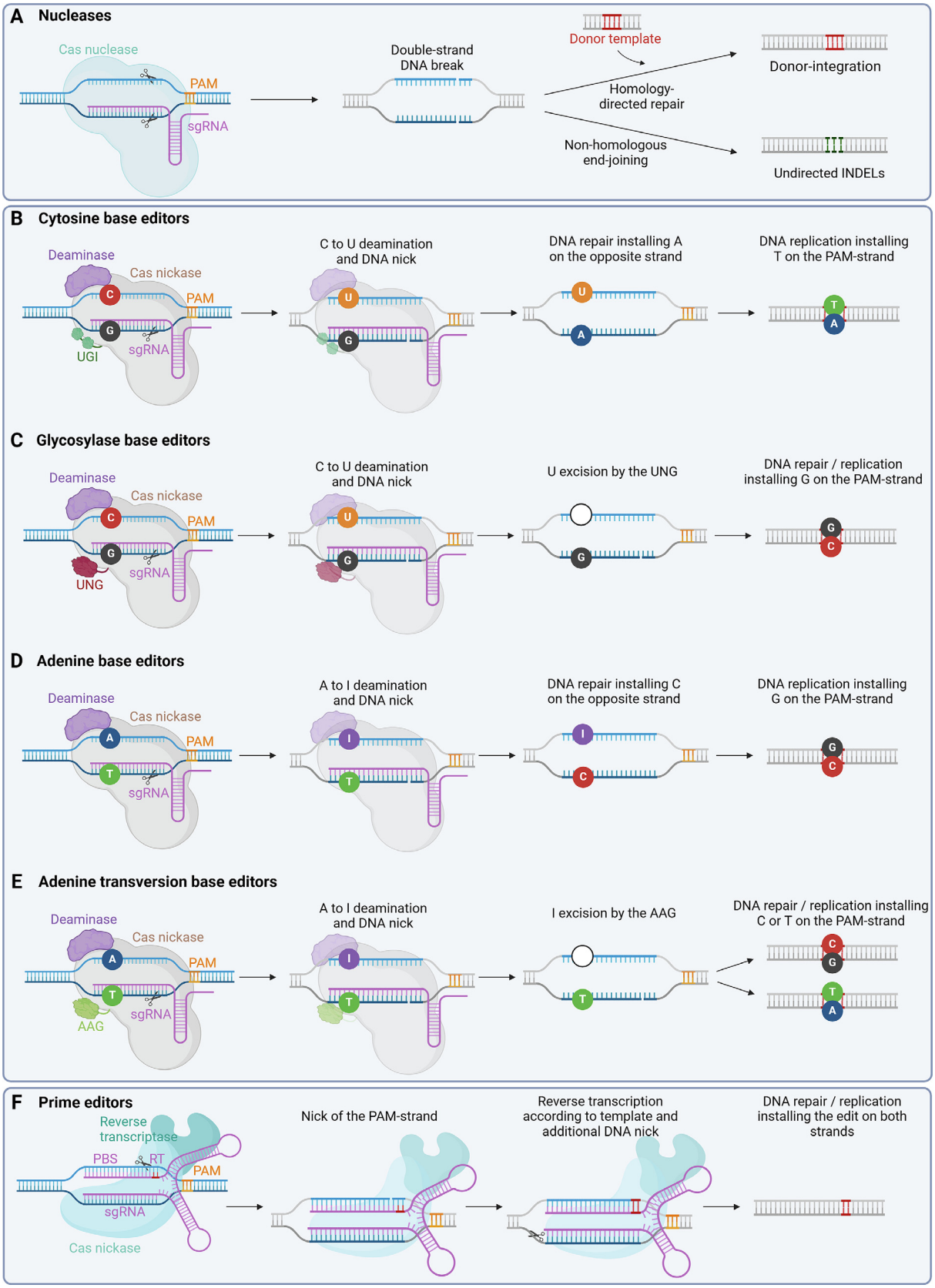
2. CRISPR-Cas9 based genome editing strategies

Originally, CRISPR-Cas9 was found as part of the adaptive immune system of prokaryotes and responsible for recognition and cleavage of foreign nucleic acids (Jiang & Doudna, 2017; Jinek et al., 2012; Mojica et al., 2005). During this process, the Cas ribonucleoproteins function as both sequence-specific targeting proteins and nucleases. Cas ribonucleoproteins target pathogenic nucleic acids, so-called spacer sequences, which are derived from previously encountered pathogens and subsequently encoded in the prokaryotic genome (Song & Koo, 2021). The recognition of target DNA is based on specific bi-interaction modes, i.e. protein-DNA and RNA-DNA base pairing (Zhou & Yao, 2023). Additionally, a suitable protospacer adjacent motif (PAM) flanking the target sequence on the foreign DNA is necessary for binding and cleavage; as the spacer sequence-encoding host DNA is lacking this PAM, it is protected against damage by Cas nucleases (Anzalone et al., 2020; Jinek et al., 2012). SpCas9, one of the first and most commonly used Cas proteins, recognizes NGG PAMs, which restricts its applicability substantially. By alteration of the PAM specificity as well as nuclease type and activity, CRISPR systems are programmable to precisely recognize a wide range of different DNA/RNA sequences (Pickar-Oliver & Gersbach, 2019). That, along with its applicability within various cell types, has made CRISPR-Cas a substantial tool in research and medicine over the last decade and spawned an enormous amount of potential applications (Wang & Doudna, 2023). Current CRISPR-Cas9 genome editing tools can be categorized in three classes, which are nucleases, base editors, and prime editors (Fig. 1).

2.1. Cas nucleases

Since its discovery in 2012, the Cas9 nuclease derived from *Streptococcus pyogenes* (SpCas9), is one of the most extensively utilized gene editing tools showing evident potential for treatment of a variety of genetic diseases (Asmamaw & Zawdie, 2021; Chavez et al., 2023). Depending on their targeting properties, nucleases are categorized into two classes, which are class-I and class-II. In class-I systems, multiple protein units are involved in recognition and cleavage of the nucleic acids, while in class-II systems the effector domains are located on a single protein (Anzalone et al., 2020; Hidalgo-Cantabrana & Barrangou, 2020; Wang, Zhang, & Feng, 2020). The type-II nuclease Cas9 consists of two endonuclease domains RuvC and HNH. The HNH domain is responsible for target recognition and operates dual RNA-guided. Thus, it binds the CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) forming a ribonucleoprotein complex (Koonin et al., 2017). The two crRNAs, sometimes also referred to as dual guide RNA, can be consolidated into one single guide RNA (sgRNA), allowing more directed applications for biotechnological use (Tan et al., 2022). When aligned in the ribonucleoprotein complex, the HNH domain cuts the RNA bound target strand. Simultaneously, the RuvC domain cuts the opposite strand, generating a precise double-strand break (DSB) within the protospacer of the target sequence (Liu, Li, & Li, 2023). The DSBs stimulate DNA repair mechanisms in the cell that can be categorized in two groups: non-homologous end-joining (NHEJ) and homology-directed repair (HDR). Upon NHEJ, the two DNA strands are re-ligated with random creation of small insertions or deletions (INDELs). Overall, NHEJ is more efficient than HDR, but also more error-prone. INDEL formation can disrupt the open reading frame with subsequent change of the amino acids and may potentially generate a premature stop-codon (Doudna, 2020; Liu & Olson, 2022; Olson, 2021). In contrast, HDR is more directed and replaces DNA sequences according to a specific DNA template sequence (Pickar-Oliver & Gersbach, 2019). Unfortunately, HDR relies on proteins that are mainly expressed during the S/G2-phase of the cell cycle and requires actively dividing cells for the repair mechanism (Nambiar et al., 2022; Miyaoka et al., 2016). Thus, HDR is only possible in a subset of cell types and not feasible in terminally differentiated and postmitotic cells like cardiomyocytes (Fig. 1A) (Zhang et al., 2024).

Besides the wildtype SpCas9, there are several engineered variants carrying strategic mutations, e.g. nickase Cas9 (nCas9) or dead Cas9 (dCas9). These variants have been modified to induce single-stranded DNA cleavage or are catalytically inactive (Jiang & Doudna, 2017). Possible applications for gene editing with nucleases have been further expanded by the development of Cas9 variants with versatile preferences in different PAMs, such as Cas9-VQR or Cas9-SpRY (Huang et al., 2022; Kleinstiver et al., 2015; Walton et al., 2020). Cas9-SpRY, developed by the Kleinstiver lab, represents a near-PAMless variant that recognizes NRN PAMs (and also NYN PAMs to a lesser extent), which substantially increases the number of editable loci (Walton et al., 2020). Furthermore, the discovery and utilization of Cas nucleases besides Cas9, e.g. Cas12 or Cas13, have expanded the genetic toolbox and its possibilities (Kordyś et al., 2021). The main advantage of Cas12 is that it creates staggered cuts distal to the PAM sequence, thereby


A Nucleases
Cas nuclease
PAM
sgRNA
Double-strand DNA break
Donor template
Homology-directed repair
Donor-integration
Non-homologous end-joining
Undirected INDELs
B Cytosine base editors
Deaminase
Cas nickase
PAM
sgRNA
UGI
C to U deamination and DNA nick
DNA repair installing A on the opposite strand
DNA replication installing T on the PAM-strand
C Glycosylase base editors
Deaminase
Cas nickase
PAM
sgRNA
UNG
C to U deamination and DNA nick
U excision by the UNG
DNA repair / replication installing G on the PAM-strand
D Adenine base editors
Deaminase
Cas nickase
PAM
sgRNA
A to I deamination and DNA nick
DNA repair installing C on the opposite strand
DNA replication installing G on the PAM-strand
E Adenine transversion base editors
Deaminase
Cas nickase
PAM
sgRNA
AAG
A to I deamination and DNA nick
I excision by the AAG
DNA repair / replication installing C or T on the PAM-strand
F Prime editors
Reverse transcriptase
PBS
RT
PAM
sgRNA
Cas nickase
Nick of the PAM-strand
Reverse transcription according to template and additional DNA nick
DNA repair / replication installing the edit on both strands

facilitating insertion of a donor DNA in a programmable manner. The main function of Cas13 is the targeting and cleavage of RNA (Wang & Yang, 2023). In summary, the variability and high efficiency of CRISPR-Cas9 combined with its easy-of-use and accessibility have made it a powerful gene editing tool, facilitating different applications in various research fields. However, this first generation of CRISPR-Cas9 gene editing is limited as NHEJ is a non-specific DNA repair mechanism and HDR is not feasible in all types of cells. Therefore, base editing technology has been developed as a second generation of gene editing.

2.2. Base editing technology

Base editing has been demonstrated to be a versatile tool for the efficient correction/editing of single nucleotide variants. BEs are fusion proteins of a deaminase and either nCas9 or dCas9, thereby avoiding DSBs (Rees & Liu, 2018). This is an important safety feature, as DSBs can result in INDEL formation and subsequent frameshift-causing mutations (Rees & Liu, 2018). BEs edit the genome within a defined editing window in relation to the PAM site of a sgRNA (Chai, Chemello, et al., 2023; Gaudelli et al., 2017; Koblan et al., 2018; Komor et al., 2016). One class of base editors are cytosine base editors (CBEs), where a cytidine deaminase is used to convert C:G to T:A base pairs via uracil intermediates. This CBE was used by Komor et al. to convert APOE4, the most common genetic risk-factor for Alzheimer's disease into the APOE3r variant in mouse cells (Komor et al., 2016). Mechanistically, the nCas9 binds to the DNA locus of interest with the corresponding sgRNA, forming the Cas-RNA-DNA complex. By local denaturation of the DNA strand, the nCas9 generates an “R-loop” exposing a small strand (~5 nucleotides) of ssDNA for deamination of cytosines (e.g., by the deaminases APOBEC, AID or CDA) (Lam et al., 2023; Porto et al., 2020). Upon DNA replication, the resulting uracil is read as a thymine, thereby creating a precise C-to-T edit (Komor et al., 2017). As uracil is rapidly excised from genomic DNA by uracil DNA N-glycosylase (UNG2), most CBEs contain uracil glycosylase inhibitors (UGIs) that increase the half-life of uracils, improving the editing efficiency (Fig. 1B). Newer approaches also use glycosylase base editors (GBEs) that are capable of converting C-to-A or C-to-G in *E. coli* and mammalian cells, respectively (Koblan, Arbab, et al., 2021; Kurt et al., 2020; Zhao et al., 2021). They consist of nCas9, a cytidine deaminase and an uracil *N*-glycosylase (UNG) that specifically excises the uracil base. At that apyrimidinic site, DNA repair processes are initiated, creating a C-to-G conversion in mammalian cells (Dong et al., 2022; Zhao et al., 2021). Although GBEs hold great potential for hereditary diseases caused by G:C mutations, currently available GBEs are limited by efficiency, specificity, and safety (Fig. 1C) (Kurt et al., 2020).

Similar to cytosine, adenine also contains an exposed amine suitable for deamination. Gaudelli et al. utilized *E. coli* tRNA-specific adenine deaminase TadA for the creation of the first adenine base editor (ABE) that deaminates adenine to inosine (Chai, Cui, et al., 2023; Gaudelli et al., 2017; Rees & Liu, 2018). The inosine pairs with cytosine and enables specific A-to-G editing. As excision of intracellular inosine is less efficient than that of uracil, no DNA repair manipulation component (like UGI) is necessary (Gaudelli et al., 2017; Porto et al., 2020). To date, there are various CBEs and ABEs with different PAM requirements and editing efficiencies (Anzalone et al., 2019; Gaudelli et al., 2017; Komor et al., 2016; Qin et al., 2020). For instance, ABEmax is an optimized, narrow-windowed ABE variant developed for precise and well predictable edits (Koblan et al., 2018). ABE8e has a wider editing window and is more processive, facilitating higher editing efficiencies but also a higher off-target editing rate (Richter et al., 2020). ABE8e has recently been developed further to ABE9e, a variant providing similar editing efficiencies as ABE8e but with a smaller editing window (Fig. 1D) (Tu et al., 2022). Usage of different Cas orthologous and engineered deaminases increases the spectrum of editable genomic segments (Liu, Zhu, et al., 2023). Tong and coworkers developed the first adenine transversion base editor (AYBE) that is capable of A-to-C and A-to-T edits with efficiencies of up to 72 % (Tong et al., 2023). They fused different ABEs to alkyladenine DNA glycosylases (AAG) that specifically excise the hypoxanthine base. That apurinic site is subsequently repaired by insertion of either a C or a T, meaning a mixed edit. The ratio of C/T insertion depends on the specific composition of the AYBE, but in general A-to-C is the preferred edit (Fig. 1E) (Chen et al., 2024; Tong et al., 2023).

The common characteristic of BEs is the promiscuous editing activity of the deaminases within the editing window, potentially leading to bystander edits (Grünewald et al., 2019). The consequences of unspecific off-target editing depend on the specific genomic locus, as editing of non-coding regions may be inconsequential. Off-target editing can be reduced by inducing strategic mutations in the TadA domain of the base editor (Richter et al., 2020). For example, introducing a V106W mutation decreased off-target editing from 29.5 % to 9.2 % (Lebek et al., 2024; Richter et al., 2020). Some approaches to reduce off-target editing result in decreased on-target editing at the same time (Park & Beal, 2019). Another successful approach to increase the editing efficiency is the utilization of double-check base editing (DBE). By introducing an active Cas9 with the same editing sgRNA as the base editor, gene copies with non-edited target sites get eliminated by the Cas9 nuclease (Xin et al., 2019). This could be especially applicable to enrich the percentage of edited cells and to erase toxic mutant copies. Summarizing, base editors are powerful gene editing tools providing high editing efficiencies for specific single nucleotide edits, but current base editors cannot perform all kinds of edits.

2.3. Prime editing technology

The major limitation of ABEs and CBEs is their inability to generate certain nucleotide edits or to induce INDELs. Prime editors (PEs) extend the editing possibilities tremendously as they can introduce all 12 possible transition and transversion changes as well as precise insertions or deletions (Anzalone et al., 2019; Molla et al., 2021). They consist of an engineered reverse transcriptase (RT) fused to nCas9 and a prime editing guide RNA (pegRNA). The pegRNA contains a sequence complementary to the target strand (sgRNA) that anneals to a target site, a scaffold for the nCas9, RT template programmed for the desired edits, and a primer binding site for binding and exposing the non-complementary, PAM-containing strand to the nickase (Kantor et al., 2020). The nicked strand is subsequently extended by the reverse transcriptase using the 3´end of the pegRNA as a template. Depending on which of the two resulting flap DNA strands hybridizes with the non-PAM containing DNA strand, the edit gets eventually integrated in the genome.

Fig. 1. Overview of CRISPR-Cas9 genome editing tools. A) CRISPR-Cas9 nucleases are guided to a specific locus in the genome, based on sequence homology with the protospacer sequence of the single-guide RNA (sgRNA). Following recognition of a protospacer adjacent motif (PAM), they perform a double-stranded DNA break. In the presence of a donor template, the DNA can be repaired by homology-directed repair with integration of the donor. The DNA can also be repaired by non-homologous end-joining (NHEJ) that can introduce small insertions or deletions (INDELs). B) Cytosine base editors consist of a deaminase that is fused to a Cas nickase and require an uracil glycosylase inhibitor (UGI). Cytosine base editors create C-to-T edits. C) Glycosylase base editors consist of a deaminase that is fused to a Cas nickase and require an uracil N-glycosylase (UNG) that specifically excises the uracil base. Glycosylase base editors create C-to-G edits in mammalian cells. D) Adenine base editors consist of a deaminase that is fused to a Cas nickase and create A-to-G edits. E) Adenine transversion base editors consist of a deaminase that is fused to a Cas nickase and require an alkyladenine DNA glycosylase (AAG) that specifically excises the hypoxanthine base. Adenine transversion base editors create A-to-C or A-to-T edits in mammalian cells. F) Prime editors are composed of a Cas nickase that is linked to a reverse transcriptase, and also require a prime editing guide RNA (pegRNA). The pegRNA consists of a sgRNA, a scaffold for the Cas nickase, a reverse transcription template (RT) containing the information of the desired edit, and a primer binding site (PBS). The template can be designed for any type of edit, including insertions or deletions.

Thermodynamically, the non-edited, perfectly complementary 5′ strand is favored, but this is also the strand preferentially degraded by cellular endonucleases when exposed. This facilitates permanent integration of the edited strand into the genome and enables programmed modification of the target region (Anzalone et al., 2019; Liu et al., 2021). Anzalone et al. introduced the first three prime editing systems in 2019. Prime editor 1 (PE1) consists of an engineered nCas9 fused C-terminally to a wildtype Moloney murine leukemia virus RT (M-MLV-RT). For the next generation (PE2) an M-MLV-RT pentamutant was utilized providing increased thermostability, higher substrate affinity and about threefold higher editing efficiency. Prime editor 3 (PE3) further increased the editing efficiency by nicking also the non-edited strand either in parallel (PE3) or after the edit (PE3b), therewith stimulating DNA repair mechanisms (Fig. 1F) (Anzalone et al., 2019). However, even though PEs can perform all types of edits, their editing efficiency is much lower than that of BEs (Liu & Olson, 2022; Nelson et al., 2022). It is therefore imperative to take both the desired edit and the required editing efficiency for therapeutic success into consideration, when developing the gene editing strategy. For some diseases, also lower editing efficiencies are sufficient to achieve therapy (e.g., ~15 % for Duchenne muscular dystrophy) (Olson, 2021).

2.4. Transcriptional and epigenetic regulation with CRISPR-Cas9 technology

The dCas9 lacking the nuclease activity can also be deployed for regulating transcriptional and epigenetic processes in a non-mutagenic manner at a specific genomic locus. Qi and coworkers developed the CRISPR interference (CRISPRi) system in 2013 (Qi et al., 2013). It consists of a dCas9 and a sgRNA that bind to a promoter or open-reading-frame. Transcriptional downregulation of the targeted gene is achieved by sterical inhibition of transcriptional elongation, RNA polymerase binding or transcription factor binding (Bikard et al., 2013; Larson et al., 2013; Qi et al., 2013). In a next generation, the CRISPRi system was fused to a transcriptional repressor domain to silence the expression of multiple genes downstream of the targeted promoter (Gilbert et al., 2013). For CRISPRa, the dCas9 is fused to transcription activation domains that induces gene expression. Frequently used factors therefore are VP64, P65 or Rta. These effector proteins can also be recruited with a suitable sgRNA, facilitating a more robust gene activation in combination with the dCas9 complex (Chavez et al., 2015; Maeder et al., 2013; Perez-Pinera et al., 2013). Bikard and coworkers fused a dCas9 to the ω-subunit of an *E. coli* RNA polymerase, which is known to effectively recruit RNA polymerases and thereby activate gene expression (Bikard et al., 2013; Dove & Hochschild, 1998). Due to various improvements, CRISPRa is an efficient and versatile tool that can be applied in a variety of cell types and at specific loci (Heidersbach et al., 2023; Jensen et al., 2021; Liu et al., 2019). Additionally, dCas9 can be fused to DNA modifying enzymes (e.g., DNA methyltransferases or DNA demethylation enzymes) or chromatin modifying enzymes (e.g., histone demethylases, deacetylases or methyltransferases), thereby regulating epigenetic modifications and subsequent gene expression (Adli, 2018; Cano-Rodriguez et al., 2016; Hilton et al., 2015; Kearns et al., 2015; Kwon et al., 2017; Liu & Olson, 2022).

2.5. Delivery strategies for gene editing tools

A crucial part of all gene editing strategies is choosing the optimal delivery strategy. For ex vivo applications like editing immune cells, stem cells or epithelial cells, there are various options to deliver the CRISPR-Cas tools into the cells. Frequently used ex vivo delivery strategies include transfection/lipofection, nucleofection, and also virus-based options (Yin et al., 2017). That in combination with the non-permanent exposure of the editing components to the body makes ex vivo editing of human primary cells very promising (Gough & Gersbach, 2020; Lahr et al., 2023). However, many clinical applications require in vivo delivery. The potential methods can be categorized in three major groups, which are viral-based, non-viral-based and physical delivery strategies (Fig. 2) (Wang, Zhang, & Gao, 2020). Physical delivery, i.e. microinjection and electroporation, can deliver DNA, mRNA or proteins via penetration of the cell membrane (Tong et al., 2019). However, these strategies are not commonly used to deliver gene editing components for cardiovascular diseases.

Viral approaches include lentivirus, adenovirus, sendai virus, retrovirus, and adeno-associated virus (AAV) – all with various advantages and limitations (McClements & MacLaren, 2017). While lentivirus and AAV trigger very low immune and inflammatory response, they display limited packaging capacities and often require a dual split-virus system (and thus a higher virus dose) (Thomas et al., 2003). Adenovirus (80–90 nm diameter, 36 kb packaging capacity) or lentivirus (80–120 nm diameter, 8 kb packaging capacity) have a higher packaging capacity and quick transgene expression, but induce a high immune and inflammatory response (Tong et al., 2019; Wanisch & Yáñez-Muñoz, 2009). A common limitation of viral-based delivery strategies is the risk of genomic integration, which is lower when using AAV (18–26 nm diameter, 4.7 kb packaging capacity) (Kupatt et al., 2021). Compared to other current delivery options, AAVs balance safety and efficiency, wherefore they are commonly used for in vivo delivery and are also frequently deployed in clinical trials (Kuzmin et al., 2021). A significant advantage of AAVs is that different serotypes can be utilized to target specific tissues or organs based on their tropism (Kuang et al., 2021). The AAV serotype-9 for instance is commonly used to target cardiac tissue. Off-organ editing can be minimized by using tissue-specific promoters (e.g., a cardiomyocyte-specific troponin T promoter) to drive the expression of the gene editing components in certain cells or tissues (Chai, Cui, et al., 2023; Lebek et al., 2024; Lebek, Chemello, et al., 2023; Nishiyama et al., 2022). Unfortunately, some promoters require significantly higher doses of editing components to reach high editing efficiencies (Grosch et al., 2022). A minor drawback of AAVs is their limited packaging capacity of 4.7 kb. This problem is addressed by using a dual virus system: either a mini-Cas < 4.7 kb and the sgRNA are delivered separately with two AAVs or a split-intein trans-splicing system is used, which allows Cas splitting and delivery with two AAVs (Chemello et al., 2021; Kupatt et al., 2021). However, the editing efficiency can be limited as both parts of the split-virus system need to enter the cell to enable expression of the complete editing system. This challenge was addressed by smaller Cas variants (with usually a lower editing efficiency) that fit within one AAV (all-in-one vectors), which reduces virus dose and production costs (Liu & Olson, 2022). Newer approaches successfully deployed self-complementary AAVs that allow delivery of double-stranded DNA. As this strategy enables faster transcription and is also less prone to degradation than single-stranded DNA-based delivery, it was possible to reduce AAV doses by 20-fold. To date, this method was mainly used in the field to deliver sgRNAs (Min et al., 2020; Zhang et al., 2020).

As people often have pre-existing antibodies against the AAV capsid, systemic gene editing therapies delivered via AAV were reported to cause severe immunological responses (Argiro et al., 2024; Ertl, 2022; Hinderer et al., 2018; Koblan, Erdos, et al., 2021). Even though this can be mitigated by corticosteroid treatment, it is imperative to keep the viral dose as low as possible, which can be achieved by engineered AAV variants with an improved tissue tropism (Tabebordbar et al., 2021; Weinmann et al., 2020). MyoAAV 2 A for instance transduces cardiomyocytes with a 17-fold increased efficiency while the liver was targeted 2.5-fold less (Tabebordbar et al., 2021). Another AAV capsid, AAV-PR, was designed to target preferentially the vasculature. Ramirez and coworkers developed this first viral vector for transduction of brain pericytes and smooth muscle cells (Ramirez et al., 2023).

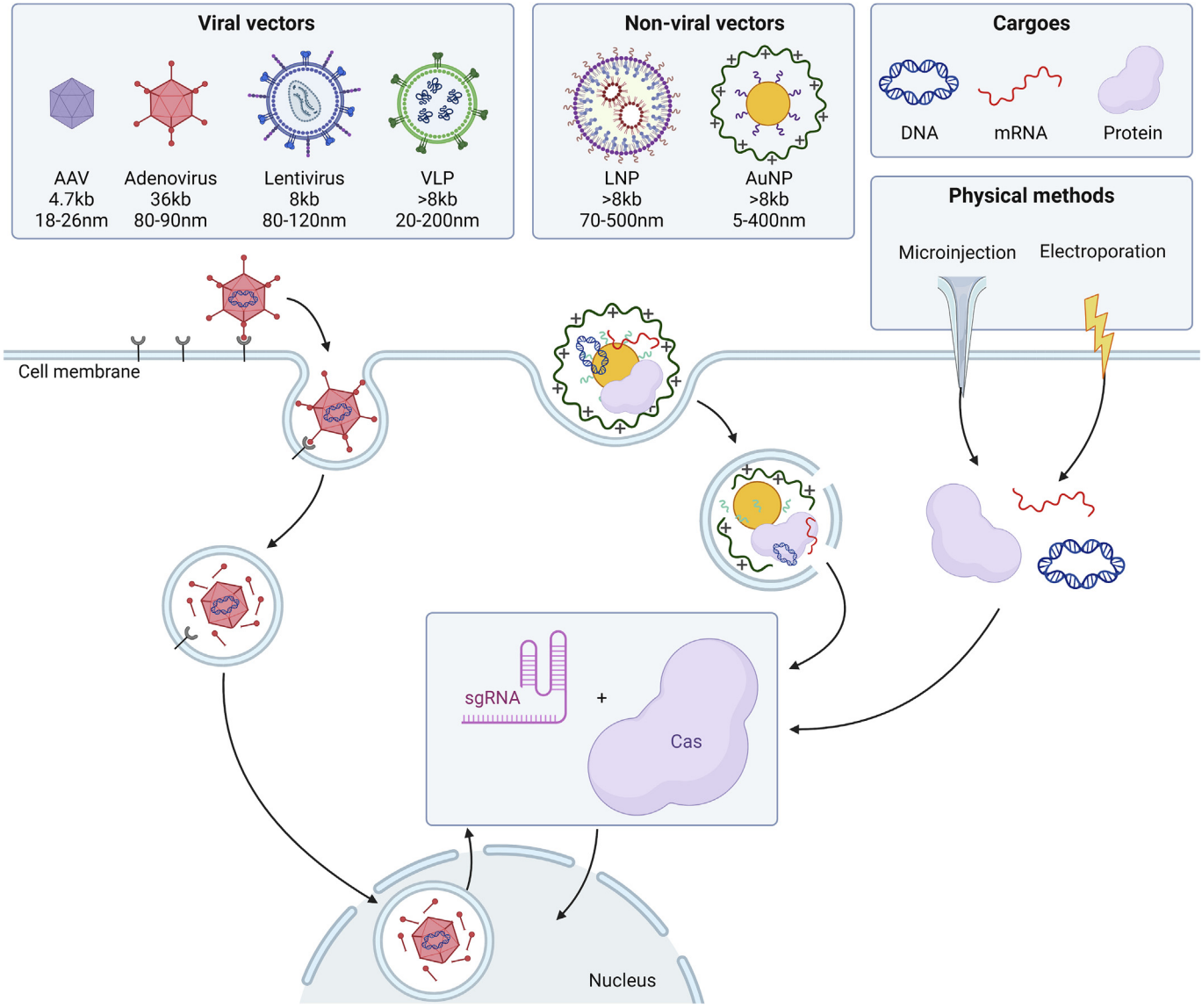


Fig. 2. Strategies for the delivery of gene editing components in vivo. Delivery strategies can be classified into three main groups: viral vectors, non-viral vectors, and physical methods. The most common viral vectors are adeno-associated virus (AAV), adenovirus, lentivirus, and virus-like particles (VLPs), and they can be used to deliver DNA. Non-viral vectors, for instance lipid nanoparticles (LNPs) or gold nanoparticles (AuNPs), can be used to deliver DNA, mRNA, and complete proteins. The size of the vectors and their respective packaging limit are indicated. Physical methods like microinjection and electroporation can be used to deliver DNA, mRNA, and complete proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Another delivery option are virus-like-particles (VLPs, 20–200 nm diameter, >8 kb packaging capacity) (Chen et al., 2023). The most extensively used virus-like-particles are lentiviral capsid proteins, consisting of two copies of the viral RNA and a capsid. As the viral RNA is not essential for assembly and cell entry, VLPs can be packaged and used for delivery of mRNA, proteins or ribonucleoproteins (Mattei et al., 2015). An additional advantage compared to AAVs is that VLPs can be produced and derived from commercially available plasmids, thereby reducing the manufacturing cost (Lyu et al., 2020).

As an alternative to virus-based strategies, the development of non-viral vectors, like lipid nanocarriers and anorganic polymeric, is in the focus of current research. The most promising approaches utilize immune-compatible lipid nanoparticles (LNPs, 70–500 nm diameter) or coated gold nanoparticles (AuNPs, 5–400 nm diameter), specially designed to suit their cargoes. In contrast to viral-based delivery, these nanocarriers also enable the transport of small proteins (Dilliard et al., 2021). As they are widely adjustable in size, shape and coating, it is possible to preferentially target certain organs or tissue regions and there is almost no restriction for the cargo size (Cheng et al., 2020; Dilliard et al., 2021; Hecker, 2016). The Siegwart lab has pioneered the development of Selective ORgan Targeting (SORT) nanoparticles delivering gene editing constructs to preferred tissues based on the charge of the coating proteins (Cheng et al., 2020). This approach induces much lower immune responses compared to virus-based delivery methods (Goell & Hilton, 2021). Another factor that strongly influences the immune response is the number of doses in which the components are delivered. Repeated administration of Cas9 in primates induced a stronger immune response than the initial administration, making it important to achieve the therapeutic goal with the first and only dose (Zhou & Yao, 2023). For instance, AAV-based delivery often outweighs the lower delivery efficiency of LNPs (especially in non-liver tissue) and is therefore a commonly used delivery strategy in preclinical and clinical gene editing studies (Ahmad, 2022; Lahlou et al., 2023; Longhurst et al., 2024). The efficient, safe, and targetable delivery of

gene editing systems in vivo is a major challenge for clinical application and needs to be further improved and developed (Lee et al., 2021; Mashel et al., 2020; Wei et al., 2020; Zhang, Qin, et al., 2021).

3. Gene editing strategies to correct genetic cardiovascular diseases

Conventional CRISPR-Cas9 gene editing strategies are designed to correct rare pathogenic mutations like small insertions/deletions or single nucleotide variants (Jiang & Doudna, 2017) (see Fig. 3). These mutations typically lead to a dysfunctional or even non-functional expression of a protein. Insertions or deletions that destroy the open reading frame often generate a premature stop codon resulting in loss-of-function. The open reading frame can be restored by exon deletion (double-cut), exon reframing through INDEL formation (single-cut), or exon skipping (single-cut or base editing), depending on the specific type of mutation (Liu & Olson, 2022). Single nucleotide variants lend toward the correction by base editing technology. Current preclinical gene editing strategies include but are not limited to Duchenne muscular dystrophy, severe combined immunodeficiency, Crigler-Najjar syndrome, sickle cell disease, beta thalassemia, cystic fibrosis, Fanconi anemia, and various kinds of cardiomyopathies (Badat et al., 2023; Lahr et al., 2023; Musunuru et al., 2021; Packer et al., 2022).

3.1. Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder affecting almost exclusively male patients due to its recessive character. The monogenic disease is caused by pathogenic mutations in the dystrophin gene resulting in a loss of functional dystrophin, which acts as a cellular shock absorber keeping myocyte integrity and stability (Chemello et al., 2023; Duan et al., 2021; Erkut & Yokota, 2022; Olson, 2021). Hence, absence or abnormal expression of dystrophin leads to degeneration of cardiac and skeletal muscle; the first symptoms are observable in early childhood and progressively worsen, until loss of ambulation and premature death due to heart failure (Guiraud et al., 2015; Padmaswari et al., 2023). Although therapeutic possibilities for treatment of DMD and symptom management have improved in the last years, to date there is no long-term curative treatment (Fortunato et al., 2021). Most mutations in the *DMD* gene are deletions of one or multiple exons with subsequent disruption of the open-reading frame. This often results in premature stop codons and truncated dystrophin proteins with rapid degradation (Li et al., 2015; Zhang, Nishiyama, et al., 2021). To restore functional dystrophin expression, specific exon skipping by antisense oligonucleotide-mediated splice mediation is one promising approach. Currently, there are four different oligonucleotide-based drugs approved by the FDA (Clemens et al., 2020; Frank et al., 2020; Lim et al., 2017; Mendell et al., 2016; Wagner et al., 2021). However, this strategy requires weekly administration via intravenous infusion.

Recent advances have also made gene engineering a promising approach for long-term correction of *DMD* mutations, allowing even specific exon excision, exon reframing, and correction of point mutations (Hanson et al., 2021; Olson, 2021). For gene editing, a wide range of tools like meganucleases, zinc-finger nucleases, transcription activator-like effector nucleases (TALENS) or CRISPR-Cas9 can be utilized (Chapdelaine et al., 2010; Lombardo et al., 2007; Yokota et al., 2007; Zhang et al., 2022). Compared with the other technologies, CRISPR-Cas9 seems to be the most promising approach due to its efficiency, genomic target specificity, and relatively good safety features. The Olson lab has been at the forefront of developing CRISPR-Cas9-based approaches to correct *DMD* mutations for almost a decade. For the first generation of gene editing strategies, they injected Cas9, sgRNA, and a HDR template into *mdx* mice zygotes to correct the *Dmd* mutation in the germline (Long et al., 2014; Yin et al., 2014). With this strategy, they generated genetically mosaic animals with up to 100 % corrected *Dmd* genes. As this approach is not feasible in human patients, they continued with editing *Dmd* in postnatal muscle cells in vivo. They used AAV9 to deliver the Cas9 and a pair of sgRNAs to skip exon 23, which resulted in restoration of the open reading frame with subsequent dystrophin expression (Long et al., 2016). The Olson lab further deployed successfully CRISPR-Cas9 gene editing to a canine model of DMD with up to 92 % restoration of dystrophin levels in the heart (Amoasii et al., 2018). Recently, Chemello et al. developed an editing strategy for DMD caused by deletion of exon 51. They utilized optimized ABEmax delivered by an AAV9 split-intein trans-splicing system to disrupt the splice donor site of *Dmd* exon 50, resulting in skipping of exon 51 and restoration of the open reading frame with functional dystrophin expression (Chemello et al., 2021). Additionally, they developed a prime

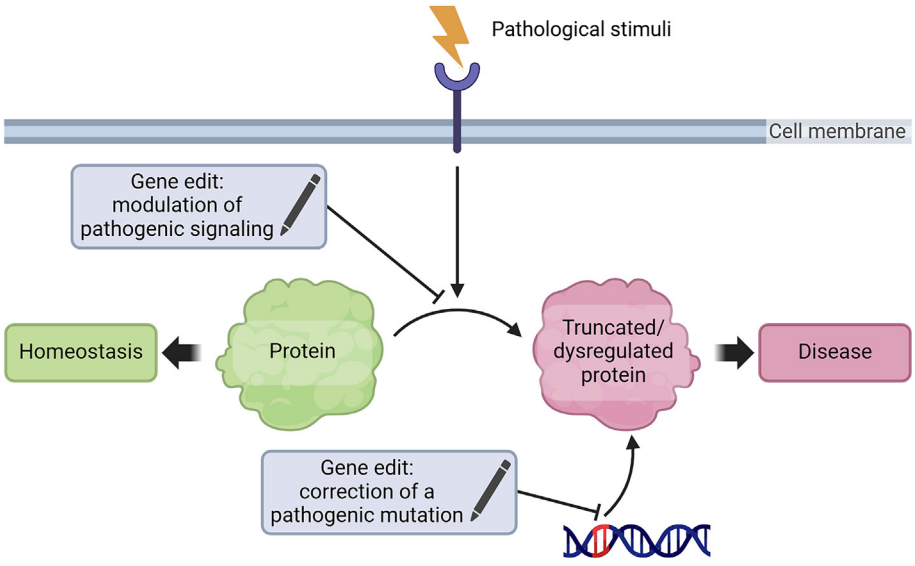


Fig. 3. Gene editing as a therapeutic strategy for non-genetic and genetic diseases. In cardiovascular disease, important effector proteins are truncated or dysregulated, thereby promoting disease development and progression. Gene editing can be deployed to either correct a pathogenic mutation or to render an effector protein resistant to pathogenic dysregulation.

editing-based reframing strategy successfully restoring the open reading frame by inserting two nucleotides in *DMD* exon 52 (Chemello et al., 2021).

In 2020, Moretti and colleagues successfully corrected a DMD pig model lacking exon 52 (Moretti et al., 2020). Using an AAV9 split-intein Cas9 with two sgRNAs flanking exon 51, they were able to restore the open reading frame leading to the expression of shortened but functional dystrophin. Notably, the time the pigs spent in an upright position and also their survival were significantly increased. Plus, isolated cardiomyocytes were less susceptible to arrhythmias ex vivo (Moretti et al., 2020).

3.2. Cardiomyopathies

Inherited cardiomyopathies will be one key area for gene editing in cardiovascular diseases. There are several entities, like hypertrophic, dilated, arrhythmogenic, non-compaction or restrictive cardiomyopathy, and also channelopathies. These disorders frequently culminate in heart failure, high arrhythmic risk, and sudden cardiac death (Watkins et al., 2011). Hypertrophic cardiomyopathy is an autosomal dominant condition characterized by increased myofilament activation, cardiomyocyte hypercontractility and excessive energy use. It can be caused by various mutations in sarcomere genes, mostly in *MYH7* or *MYBPC3* (each accounting for 25 %–30 % of HCM cases) (Chai, Cui, et al., 2023; Nguyen et al., 2020; Reichart et al., 2023). Non-ischemic dilated cardiomyopathy is a mostly autosomal dominantly inherited disease. The causative mutations are found in over 40 different genes that encode for nuclear envelope proteins (e.g., *LMNA*), the contractile apparatus (e.g., *MYH7*), membrane scaffolding proteins (e.g., *SGCD*) or proteins related to calcium handling (e.g., *PLN*) (Nguyen et al., 2020; Nishiyama et al., 2022; Watkins et al., 2011). Arrhythmogenic cardiomyopathy is inherited autosomal dominantly and affects predominantly the right ventricle. Typical mutations are in the genes encoding desmosomal proteins (e.g., desmosomal cadherins or desmoplakins) and proteins that are interacting with them. The resulting loss of desmosomal function results in cardiomyocyte death and subsequent fibro-fatty replacement of the myocardium (Nguyen et al., 2020). Non-compaction cardiomyopathy is an autosomal dominant or X-linked recessive condition caused by mutations or variants in genes encoding sarcomere (e.g., *MYH7*), *Z*-disc (e.g., *LDB3*), nuclear envelope (e.g., *LMNA*), mitochondrial (e.g., *TAZ*) or ion channel proteins (e.g., *SCN5A*), which affect compaction of the endomyocardial layer (Nguyen et al., 2020; Towbin, 2014). Restrictive cardiomyopathy, the least frequent cardiomyopathy entity, is inherited autosomal dominantly and caused by mutations in sarcomere genes or transthyretin (Nguyen et al., 2020). As many hereditary cardiomyopathies are inherited autosomal dominantly, entire families are affected with often severe clinical symptoms, which makes it imperative to develop durable and effective therapeutic strategies. CRISPR-Cas9 gene editing to correct these mutations back to wildtype would be a causative therapeutic approach.

Even though the delivery, editing efficiency, and potential immunogenicity of different editing approaches remain a challenge in the field, the feasibility of therapeutic gene editing for cardiomyopathies was already demonstrated in various disease models (Kyriakopoulou, Monnikhof, & van Rooij, 2023; Moore et al., 2023). Several hallmark studies were performed by the Olson laboratory (Chai, Cui, et al., 2023; Nishiyama et al., 2022). Nishiyama et al. demonstrated successful base editing in a mouse model of non-ischemic dilated cardiomyopathy and corrected different mutations (p.R634Q and p.R636S) in the RNA binding motif protein 20 encoding gene (*RBM20*). Homozygous mutant mice showed a reduced fractional shortening, atrial and ventricular dilation, and other heart failure phenotypes, which were significantly improved by treatment with AAV9-ABEmax-VRQR-SpCas9. The researchers achieved in vivo a cardiac editing efficiency of 66 % at the cDNA level. As they used a cardiomyocyte-specific troponin T promoter to drive the expression of the editing components exclusively in the heart, they did not detect editing in other tissue, which substantially reduced the risk of adverse effects (e.g., in the liver) (Nishiyama et al., 2022). Similarly, Grosch et al. achieved up to 87 % restoration of RBM20 defects in cardiomyocytes with different ABEs. They were able to increase the editing efficiency by utilizing AAVMYO, an AAV variant with improved tropism for cardiomyocytes (Grosch et al., 2022).

Another very elegant editing approach was developed by Chai and coworkers following a thorough and comprehensive optimization of the base editing strategy. They targeted the dominant-negative c. G1208A (p.R403Q) pathogenic variant in β-myosin (*MYH7*) that is causative for a severe form of hypertrophic cardiomyopathy with premature death. The cardiac editing efficiency achieved with AAV9-delivered ABEmax was 32.3 % at the DNA level, thereby restoring cardiac function and geometry, and doubling the lifespan of the mice (Chai, Cui, et al., 2023). Reichart et al. compared two different CRISPR-Cas9 strategies for the same mutation. First, they used ABE8e base editing to correct the pathogenic mutation with an editing efficiency of more than 70 % in ventricular cardiomyocytes. In another approach, they specifically inactivated the pathogenic p.R403Q allele with a Cas9 nuclease. Both gene editing strategies successfully prevented the development of hypertrophic cardiomyopathy (Reichart et al., 2023).

Dave and coworkers targeted the p.R14del mutation in the *phospholamban* (*Pln*) gene that was linked to clinical phenotypes of both dilated and arrhythmogenic cardiomyopathy. Using an AAV9-SaCas9-sgRNA construct, they inactivated the pathogenic allele in a humanized mouse model, thereby preventing ventricular dilation and sustained ventricular tachycardia (Dave et al., 2022).

Another concept to treat conditions caused by a loss-of-function mutation is the overexpression of the wildtype gene, as nicely shown by Kyriakopoulou et al. (Kyriakopoulou, Versteeg, et al., 2023). As arrhythmogenic cardiomyopathy can be caused by many different mutations in desmosomal genes (e.g., *PKP2*), a broader applicable therapeutic approach would be helpful (Marcus et al., 2010). Using AAV9-mediated delivery of wildtype *PKP2*, Kyriakopoulou and colleagues completely restored plakophilin 2 protein levels in mice and engineered human myocardium, which led to complete recovery of desmosomal and contractile function. They thereby provided a new therapeutic strategy for arrhythmogenic cardiomyopathy (Kyriakopoulou, Versteeg, et al., 2023).

3.3. Comprehensive approaches for genetic diseases

One major challenge in correcting pathogenic mutations is that each gene editing strategy is designed for the correction of one specific mutation. The individual frequency of such mutations is typically low, which precludes broad applicability. Hypertrophic cardiomyopathy, for example, affects 0.2 % of the population, and causative inherited mutations can be identified in 60 % of all cases (Abbas et al., 2024; Maron et al., 1995). However, there are over a dozen potential genes harboring the mutation, and there are even many different mutations described within the same gene (Marian, 2021; Teekakirikul et al., 2013). As the development of gene editing strategies incurs substantial economic costs, it is challenging for the health system to pursue approaches that are only applicable for a small subset of patients (Witkowsky et al., 2023). In fact, the costs for the genetic therapy of one patient is currently up to 3.5 million dollars. A substantial part of these costs is the design and development of the editing strategies and the testing in clinical trials (Witkowsky et al., 2023). If a therapeutic strategy would be broadly applicable, these costs could be borne by more patients, which would reduce the costs per dose. In addition, standardized large-scale-production would be possible, which is more efficient and would further reduce the costs. Thus, there is a significant need for more comprehensive gene editing strategies that could be deployed for more than a single mutation.

One approach could be to ablate pathogenic alleles (e.g., with nucleases or by introducing a premature stop codon), which could be

particularly amenable for dominant-negative mutations within the same gene. Another major group of cardiomyopathies is caused by mutations leading to haploinsufficiency of the gene with insufficient levels of functional protein being expressed. Prominent examples are loss-of-function mutations in the *TTN* or *LMNA* genes (Tharp et al., 2019; Wang & Dobreva, 2023). CRISPR-Cas9-based approaches could be used to increase the expression of the wildtype alleles, for example by installing additional regulatory elements using prime editing or by using CRISPRa technology. Another comprehensive strategy for mutations causing *TTN* haploinsufficiency could be activating the *Cronos* promoter (Repetti et al., 2019). *Cronos* is a conserved internal promoter and active in the human myocardium. It drives the expression of a shorter but functional titin isoform, which could compensate the haploinsufficiency of a *TTN* mutation (Repetti et al., 2019; Zou et al., 2015). In some genes, there are hotspots with several described mutations close to each other. They could be covered and corrected with a comprehensive prime editing approach, where the reverse transcription template is programmed for the natural wildtype sequence. Instead of correcting one specific mutation, it would also be possible to silence common pathogenic signaling that is induced by several different mutations, which would broaden the applicability of one gene editing strategy. Notably, this concept can also be deployed for common non-genetic disorders, for example by modulating pivotal stress-responsive kinases in the heart (Lebek et al., 2024; Lebek, Chemello, et al., 2023; Musunuru et al., 2021).

4. Gene editing strategies to silence common pathogenic signaling cascades

4.1. Editing *CAMK2D* in the failing heart

The multifunctional Calcium/calmodulin-dependent protein kinase IIδ (CaMKIIδ), encoded by the *CAMK2D* gene, is a central regulator for myocardial function and energetic, and mediates various stress-responsive mechanisms in the heart (Beckendorf et al., 2018; Duran et al., 2021). However, upon chronic dysregulation (e.g., overactivation), CaMKIIδ turns deleterious and promotes diseases like heart failure, ischemia/reperfusion injury (IR), arrhythmias, or alcoholic cardiomyopathy (Anderson et al., 2011; Mustroph et al., 2018). Mechanistically, disturbed CaMKIIδ signaling has been implicated in impaired cardiac excitation-concentration coupling with dysregulation of cellular sodium and calcium homeostasis, inflammation, apoptosis, fibrosis, and dysregulations of the cardiac transcriptome via histone deacetylase 4 (Backs et al., 2006; Backs et al., 2009; Bers, 2008; Erickson, 2014; Fischer et al., 2014; Hegner et al., 2023; Lebek et al., 2020; Lebek et al., 2021; Lebek, Chemello, et al., 2023; Luo et al., 2013; Mustroph et al., 2018; Nassal et al., 2020; Neef et al., 2010; Pellicena & Schulman, 2014) This makes modulation of CaMKIIδ activity a promising approach for heart disease therapy (see Fig. 3).

Interestingly, CaMKIIδ is subject to posttranslational modification, which releases the catalytic domain from the autoinhibitory region resulting in sustained enzyme hyperactivation and cardiac disease (Erickson et al., 2008; Erickson et al., 2013; Hegyi et al., 2021). One main modification is autophosphorylation at threonine-287 that has been linked to various cardiac disease entities (Lebek, Caravia, et al., 2023; Wehrens et al., 2004). As this site is encoded by an ACT codon, we reasoned that ABE technology could perform a single nucleotide edit to a GCT codon that is recognized as a phospho-resistant alanine (Lebek, Caravia, et al., 2023). After a thorough screening of several sgRNAs, deaminases, and Cas9 variants, we identified a *CAMK2D* gene editing strategy that rendered CaMKIIδ phospho-resistant. This editing approach was delivered to the mouse germline to generate the T287A CaMKIIδ mouse model. These mice were normal under conditions of homeostasis. However, when subjected to severe transverse aortic constriction (≙ heart failure model), CaMKIIδ phospho-resistant mice were protected from heart failure-related mortality, impaired cardiac function, dysregulated cardiac transcriptome, myocardial apoptosis, and subsequent fibrosis (Lebek, Caravia, et al., 2023). Human cardiomyocytes derived from edited induced pluripotent stem cells (iPSCs) were protected from deterioration of cellular calcium homeostasis following chronic β-adrenergic stress. Based on this initial work, we hypothesized that *CAMK2D* editing could also be beneficial postnatally in adult mice and in other cardiovascular disease entities. Therefore, we developed a second editing approach to render CaMKIIδ resistant to oxidative activation, which was suspected to confer cardioprotection against a high burden of oxidative stress, as it is the case following ischemia/reperfusion injury (IR). This was more challenging as the oxidative activation sites of CaMKIIδ are represented by two methionines at positions 281 and 282 and it was unclear which editing pattern is the most beneficial (Lebek, Chemello, et al., 2023). Therefore, we characterized several *CAMK2D*-edited human iPSC-cardiomyocytes following in vitro simulated IR and found that a broad-windowed editing pattern with an extra bystander edit (MMH281/282/283VVR) conferred the highest degree of cardioprotection. Using an AAV9 delivery system, we then administered the *Camk2d* editing components to adult mice after IR. Notably, edited mice recovered cardiac function, showed an improved cardiac transcriptome, and less cardiac apoptosis and fibrosis (Lebek, Chemello, et al., 2023). Moving toward potential clinical translation, we then generated a humanized *CAMK2D* knockin mouse model to replace the genomic sequence encoding the entire regulatory domain with the human sequence. This enabled deployment of the editing strategy optimized for the human genome in vivo (Lebek et al., 2024). Local intracardiac injection and use of an engineered myotropic AAV (MyoAAV 2 A) enabled efficient delivery of the editing components at a very low virus dose of 1.5×10^{11} vg/kg body weight. This further optimized *CAMK2D* editing approach post-IR in humanized mice resulted in recovery of cardiac function, improved exercise capacity on the treadmill, and substantially decreased myocardial fibrosis, which was otherwise observed in injured non-edited mice (Lebek et al., 2024).

Modulation of CaMKIIδ activity as a therapeutic approach for heart disease was not new and in fact there are several CaMKIIδ inhibitors under preclinical considerations (Lebek et al., 2018; Mustroph et al., 2020; Neef et al., 2017; Neef et al., 2018; Pellicena & Schulman, 2014). However, there is still no such substance available for patients as traditional compound-based pharmacological approaches face several limitations and challenges that preclude clinical translation. The ubiquitous expression of CaMKII, the presence of 4 different isoforms (CaMKIIα, β, γ, and δ), and their crucial role in vital processes in organs other than the heart require specific inhibition of the δ-isoform, which is the main isoform in cardiac disease (Nassal et al., 2020). Outside the heart, the multifunctional CaMKII is involved in memory formation through long-term potentiation, hepatic glucose production and insulin signaling, vascular smooth muscle cell function, skeletal muscle function, cell cycle progression and fertility (Ataei et al., 2015; Beckendorf et al., 2018; Nicole & Pacary, 2020). Notably, also the δ-isoform has been demonstrated to be expressed in the brain and involved in memory and learning processes (Zalcman et al., 2018). This means that global or isoform-unspecific CaMKII inhibition may potentially cause severe adverse effects. The allosteric inhibitor KN-93 has been shown to improve cardiac function, but shows off-target effects on ion channels, like potassium channels (Nassal et al., 2020; Pellicena & Schulman, 2014). Other preclinical CaMKIIδ inhibitors were limited regarding target specificity and efficiency, and autocamtide-2-related inhibitory peptides showed a poor bioavailability (Pellicena & Schulman, 2014). ATP-competitive inhibitors like Hesperadin, RA608 or RA306 also inhibit also other kinases, as their IC_{50} for CaMKIIδ is only slightly higher than for some other isoforms (Beauverger et al., 2020; Mustroph et al., 2020; Nassal et al., 2020; Pellicena & Schulman, 2014). Plus, even an optimal CaMKIIδ inhibitor would mean an additional pill in the daily treatment regimen and relies on patients' compliance, which decreases with every extra pill (Anderson & Funnell, 2000; Gupta et al., 2017; Kulkarni et al., 2006).

We think precise and permanent modulation of *CAMK2D* using CRISPR-Cas9 gene editing addresses these limitations and challenges. Off-target effects on other enzymes or ion channels can be minimized by carefully designing an optimal editing strategy for *CAMK2D*. Indeed, we detected an over 2000-fold specificity toward *CAMK2D* compared to other isoforms for selected gene editing approaches (Lebek, Caravia, et al., 2023). Plus, using a tissue-specific promoter (e.g., the cardiomyocyte-specific troponin T promoter) to drive the expression of the gene editing components exclusively in cardiomyocytes prevents editing in other tissues, which substantially reduces the risk of adverse effects. As CRISPR-Cas9 gene editing only requires a single dose that results in a permanent edit, this approach does not rely on patients' compliance for medication intake. Notably, the limitations and challenges of CaMKIIδ inhibitors are also often observed with pharmacological compounds targeting other proteins. Thus, CRISPR-Cas9 gene editing to disrupt pathological signaling could also be transferred to other common diseases above and beyond the heart (see Fig. 3).

4.2. Gene editing strategies targeting hypercholesterolemia

In the field of hypercholesterolemia, gene editing is being deployed to generate loss-of-function mutations in the proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 is produced in the liver and is a key regulator in the lipid metabolism and an important therapeutic target for hypercholesterolemia (Nishiga et al., 2022). High levels of PCSK9 prevent uptake of LDL cholesterol in the hepatocytes, thereby increasing blood levels of LDL cholesterol and favoring cardiovascular diseases (Zhang, 2020). Currently, there are three monoclonal antibodies and one small interfering RNA for PCSK9 inhibitory therapies (Bao et al., 2024). Notable, these drugs need to be administered at least biannually up to biweekly. As an alternative long-term treatment strategy, Ding et al. utilized an AAV-CRISPR-Cas9 system to edit *Pcsk9* gene in the mouse liver. They observed approximately 50 % INDEL formation with disruption of the open-reading-frame and subsequently decreased PCSK9 protein levels. At the same time, hepatic LDL receptor levels increased and plasma LDL cholesterol levels decreased about 40 % (Ding et al., 2014). Since then, several preclinical trials demonstrated successful reduction of PCSK9 levels in mice and non-human primates (also with ABE technology) (Davis et al., 2023; Musunuru et al., 2021; Rothgangl et al., 2021; Wang et al., 2021). With editing efficiencies of up to 60 % in the monkey liver and concomitantly up to 60 % reduced LDL cholesterol levels, this approach seems to be at least as effective as currently used drugs in the clinic (Musunuru et al., 2021). Other approaches successfully utilized zinc-finger proteins for durable silencing of the *Pcsk9* gene in mice (Cappelluti et al., 2024).

With Verve-101, the first CRISPR-Cas9-based treatment strategy targeting PCSK9 is already being tested in a clinical trial (NCT05398029). Notably, a single infusion of Verve-101 reduced LDL cholesterol levels by 39–48 % in patients with familial hypercholesterolemia (Hooper et al., 2024; Horie & Ono, 2024). Unfortunately, one patient experienced increased alanine transaminase levels, a biomarker for liver disease, as well as low blood platelets, wherefore he was hospitalized despite being asymptomatic (Bayer, 2024). Even though these abnormalities resolved independently within a few days, Verve Therapeutics has halted the enrollment of study participants (Hooper et al., 2024).

4.3. Gene editing strategies for ATTR amyloidosis

Transthyretin (TTR) is a physiological transport protein for thyroxine and retinol and is synthesized predominantly in the liver. Misfolding of the TTR into pathogenic ATTR amyloid fibrils that gradually accumulates in neurons and cardiomyocytes causes severe polyneuropathy and cardiomyopathy (Ioannou et al., 2023; Shahzad et al., 2022). Cardiac ATTR amyloidosis leads to heart failure and can be diagnosed in up to 15 % of patients hospitalized for heart failure with preserved ejection fraction (Porcari et al., 2023; Shahzad et al., 2022). Current treatment strategies for ATTR amyloidosis (e.g., tafamidis or diflunisal) aim for stabilization of the TTR in its tetrameric form to prevent amyloid formation (Maurer et al., 2018). However, these strategies confer limited therapeutic benefits, are associated with adverse effects, and require long-term administration (Urits et al., 2020). ATTR amyloidosis is caused by acquired pathogenic processes associated with aging or hereditary genetic *TTR* mutations (Ioannou et al., 2023). To date, over 130 different pathogenic mutations are reported, making it difficult to selectively target and correct each individual mutation (Porcari et al., 2023). Therefore, utilizing CRISPR-Cas9 to permanently knockdown of *TTR* is a promising new approach. Several preclinical trials demonstrated successful long-term treatment ATTR amyloidosis with this strategy without overt adverse effects caused by *TTR* knockdown (Rapezzi et al., 2022). Gillmore and coworkers developed an intravenous CRISPR-Cas9-based therapy (NTLA-2001), where the gene editing components were administered in a single dose using LNPs with high tropism for the liver. With that strategy, they achieved a mean reduction of TTR levels by up to 87 % in patients with hereditary ATTR amyloidosis (Gillmore et al., 2021).

4.4. Challenges and limitations of silencing pathogenic signaling using gene editing

Besides the major advantages like high specificity and efficiency as well as sustained therapeutic benefits, CRISPR-Cas9 gene editing faces several challenges that need to be considered. Especially when introducing a protective mutation into the genome, it is imperative to critically determine and assess a suitable target. Genes and proteins have been evolved during evolution and have important functions in physiological and homeostatic conditions. However, when homeostasis is disturbed, several of these effector proteins are dysregulated and cause and perpetuate pathology. Especially disease mechanisms related to aging and cardiovascular diseases, which are typically found in older individuals, might have escaped evolution. Precise fine-tuning and prevention of protein dysregulation might be superior compared to complete ablation of an effector protein as physiological functions are preserved. This can be achieved by rendering proteins resistant to post-translational modification, which requires careful assessment of the resulting edit and the new amino acid (Lebek et al., 2024; Lebek, Caravia, et al., 2023; Lebek, Chemello, et al., 2023). Changes in the charge of an amino acid or substantial steric differences may alter the secondary and tertiary structure of the protein with unpredictable effects on protein function. One previously discussed example was the replacement of a threonine with an alanine to prevent overactivation of CaMKIIδ (Lebek, Caravia, et al., 2023). Notably, the structure of alanine is very similar to threonine but is lacking the hydroxyl-group, which rendered CaMKIIδ phospho-resistant.

CRISPR-Cas9 technology introduces a permanent edit in cardiomyocytes that warrants also long-term safety studies. However, a permanent edit could be favourable for chronic diseases, where a pathomechanism is perpetuated over many years. Another potential caveat is the risk for off-target and off-organ editing, which needs to be carefully considered. Both can be minimized by using optimized gene editing strategies (including engineered base editors like ABE8e-V106W or ABE9e) or tissue-specific promotors to drive the expression of the editing components (Lebek et al., 2024; Tu et al., 2022). Permanent expression of the gene editing components may increase the risk for off-target editing and may also cause immunological effects (Park & Beal, 2019). Therefore, there are different strategies to deliver active CRISPR-Cas9 components only temporally. This can be achieved by fusion to a destabilization domain that allows conditional expression of the gene editing components (Senturk et al., 2017). Other approaches to control Cas activity are based on nuclease splitting or chemically

controlled alteration of the protein stability (Dow et al., 2015; Jiang et al., 2017; Li et al., 2021; Maji et al., 2017; Zetsche et al., 2015). A big challenge in the field is optimizing the delivery strategies. AAVs are frequently used and enable highly efficient in vivo editing but are associated with potential adverse effects (as discussed above). Further engineered myotropic AAVs (e.g., MyoAAV 2 A) allow substantial reduction of the virus dose and there is also effort toward non-viral based delivery strategies (Dilliard et al., 2021; Lebek et al., 2024; Tabebordbar et al., 2021). Identifying the optimal timepoint for administering the editing components will be critical as gene editing cannot confer therapeutic benefits once the cardiomyocytes are gone. Therefore, preventative editing strategies (e.g., for patients at high risk for a heart attack) could also be considered.

5. Conclusion

CRISPR-Cas9 gene editing is an advanced approach for the treatment of various human diseases. Especially base editors allow targeted, specific, and efficient editing. While gene editing holds great promise for the correction of hereditary diseases, it can also be deployed to silence common pathogenic signaling cascades with broad applicability. Especially when targeting a wildtype gene to disrupt a common pathomechanism, it will be critical to determine the optimal gene to edit for each patient cohort. This depends on the main pathomechanisms of the disease, the editability of the respective genes, and the additional functions of the proteins that may not necessarily be pathologic. High target- and organ-specificity can be achieved by optimized editing tools and tissue-specific promoters, respectively, which substantially reduces the risk of adverse side effects. CRISPR-Cas9 gene editing offers a permanent intervention with sustained therapeutic benefits and does not rely on patients' compliance to take medication, which might be especially interesting for chronic disorders. Thus, CRISPR-Cas9 gene editing represents a next-generation therapeutic approach and might overcome common limitations and challenges of traditional compound-based therapies.

CRediT authorship contribution statement

Anna-Maria Lauerer: Writing – original draft, Visualization, Conceptualization. **Xurde M. Caravia:** Writing – review & editing. **Lars S. Maier:** Writing – review & editing. **Francesco Chemello:** Writing – review & editing. **Simon Lebek:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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