Trends in Molecular Medicine

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Review

CRISPR Craze to Transform Cardiac Biology

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Clustered regularly interspaced short palindromic repeats (CRISPR) has revolutionized many research areas and has rapidly become the gold standard in genome editing by outrivaling all other available tools. Its unprecedented versatility creates the opportunity to modify any aspect of gene regulation. Even though the cardiac field is starting to appreciate the potential of CRISPR, many applications to study cardiac biology and disease so far have remained untouched. In particular, CRISPR-based strategies that act independent of the homology-directed repair pathway could help circumvent issues of modifying the genome of postmitotic cardiomyocytes, which is currently limiting its utility in the heart. Here, we review current applications and future potential for the use of CRISPR to study cardiac biology and disease.

Highlights

CRISPR is an easily accessible, versatile and affordable genome editing tool. It can be used to modify basically any aspect of gene regulation both *in vitro* and *in vivo*.

Novel knock-in and knockout animal models can be generated within one generation using CRISPR-based approaches.

Pathogenic variants can now easily be reverted or introduced in human induced pluripotent stem cells, yielding isogenic controls.

The modular nature of the CRISPR system enables one to fuse it to essentially any effector protein, thereby modulating gene function.

Currently, many CRISPR-Cas applications remain unexplored for cardiac biology.

Genome Editing Tools to Tackle Cardiac Disease

Over the past decades, functional genomics has advanced our understanding of cardiac biology and disease. Yet, cardiac disorders are among the leading causes of morbidity and mortality, according to the World Health Organization information on cardiovascular disease (https://www.who.int/cardiovascular_diseases/en/). Patients suffering from these disorders frequently show changes in their genetic and **epigenetic** (see Glossary) landscape. However, the molecular pathways affected by these transformations are often poorly understood. A better understanding of cardiac biology is therefore necessary for the identification of disease-driving mechanisms and for the development of novel therapeutics.

In the last two decades, the field of **genome editing** has advanced at great speed, yielding easily accessible, versatile, and affordable tools to study biology and disease. In particular, the CRISPR system has developed into a widely applied technology to modulate gene function *in vitro* and *in vivo* [1–5]. The CRISPR genome editing tool, as we know it today, consist of the CRISPR-associated protein Cas9 from *Streptococcus pyogenes* (SpCas9), which is an RNA-guided nuclease that is directed to a specific genomic region by a single guide RNA (sgRNA) [6–8]. The only prerequisite for proper binding of the Cas–sgRNA complex is the presence of a **protospacer adjacent motif** (PAM) that is recognized by the PAM-interacting domain of the Cas9 protein [9,10]. Upon binding, Cas9 creates a nick on the complementary and noncomplementary DNA strand, thereby creating a double-strand break (DSB). Recently, a number of reports have revealed that Cas9 tends to leave a single nucleotide overhang four nucleotides distal of the PAM sequence, opposing the general idea that a blunt cut is created [11,12]. The DSB created by Cas9 will subsequently be repaired by endogenous repair mechanisms such as **nonhomologous end-joining** (NHEJ) or **homology-directed repair** (HDR) (Figure 1). Importantly, researchers have demonstrated that the HDR pathway can be used to insert any genomic sequence of interest into the genome of the host by supplying an exogenous DNA sequence with homology arms [13–17]. The latter can be used to knock in loxP sites, fluorescent proteins, **SNP**, or other regulatory sequences (Figure 1). Detailed descriptions of the CRISPR system can be found elsewhere [18,19].

Also for the cardiac field, genome editing using CRISPR has accelerated the generation of new insights into physiological and pathological cardiac biology. Here, we provide an overview of

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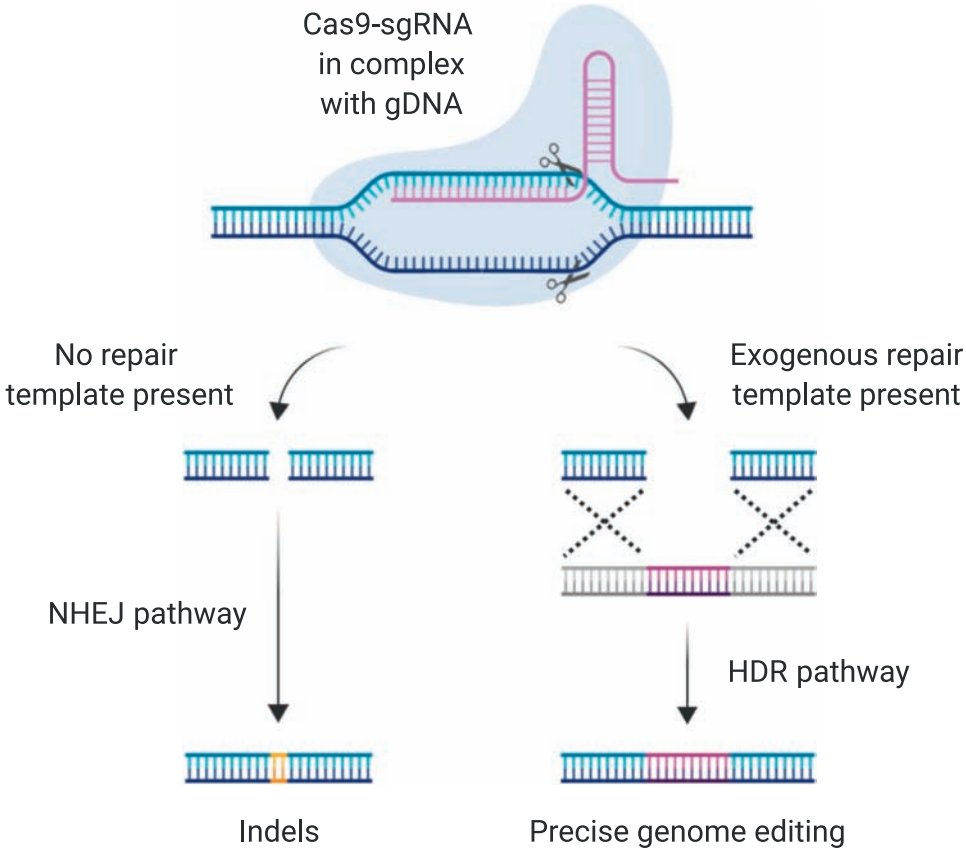

Figure 1. Mechanisms to Repair CRISPR-Induced DSBs. The sgRNA binds to the Cas nuclease, directing it towards specific genomic regions. Upon localization, the Cas protein creates a DSB that is repaired via the NHEJ or HDR pathway. NHEJ-mediated repair occurs throughout the cell cycle and is error prone in the absence of a repair template, ultimately resulting in small insertions or deletions at the cut site, thereby affecting gene function. The HDR pathway, in contrast, is a high-fidelity repair mechanism that utilizes an endogenous (e.g., sister chromatid) or exogenous DNA template to accurately repair the DSB. This property can be used to incorporate DNA elements of foreign origin, such as nucleotide variants, tags and loxP sites. Abbreviations: DSB, double-strand break; gDNA, genomic DNA; HDR, homology-directed repair; Indels, insertions/deletions; NHEJ, nonhomologous end-joining; sgRNA, single guide RNA.

the studies that have applied CRISPR to better understand cardiac disease mechanisms and list anticipated developments that further optimize and broaden the use of the CRISPR toolbox in the cardiac field.

The CRISPR Toolbox Applied in Cardiac Biology

In Vivo CRISPR-Cas Applications

CRISPR-Cas has been exploited to alter gene function in somatic cells by delivering both Cas9 and a sgRNA to a desired cell population. Routinely, researchers utilized **cardiotropic adeno-associated virus** (AAV) constructs to specifically deliver components of interest to the heart (see Clinician's Corner). For example, several groups demonstrated that AAV-mediated delivery of Cas9 and a sgRNA could restore dystrophin expression in the heart in models of Duchenne muscular dystrophy; a disease characterized by lethal degeneration of cardiac and skeletal muscle (Figure 2A) [20–26]. Many of the above-mentioned studies utilized a dual-vector approach in which the SpCas9 (4.2 kb) and the sgRNAs were delivered to the heart by separate AAV constructs [20,24–26]. This allows one to easily modify the ratio of SpCas9 and sgRNA, which

Glossary

Adeno-associated virus: small virus often used for therapeutic purposes because of its lack of pathogenicity. Multiple serotypes exist varying in their capsid protein composition and tropism.
Cardiotropic: attracted to and functions in the heart.
CTCF looping sites: specific DNA sequences bound by CTCF proteins. Upon binding, these proteins bring two DNA strands together, thereby forming chromatin loops. The latter are essential in the regulation of gene expression.
Enhancers: short regulatory DNA sequences located upstream and downstream of an associated gene. Transcription factors can bind to these sequences, consequently enhancing the expression of the associated gene.
Epigenetics: includes all reversible processes (e.g., histone modifications and chromosome looping) that affect gene expression but do not change the genetic code.
Genome editing: controlled manipulation of the genetic code of a cell or animal. Genomic regions of interest can be deleted, inserted, or replaced.
Homology-directed repair: process by which a cell repairs a DNA DSB in the presence of a repair template (e.g., sister chromosome or exogenous DNA strand). Only occurs during the S and G2 phases of the cell cycle.
Hypertrophic cardiomyopathy: disease in which the heart muscle becomes thickened.
Intraperitoneal injection: method commonly used to inject a substance (e.g., virus) into the body cavity of animals.
Mosaicism: indicates that an individual, originating from a single fertilized egg, is composed of multiple genotypes. For example, CRISPR-mediated genome editing may only affect a subpopulation of cells, introducing multiple genotypes.
Nonhomologous end-joining: error-prone process by which a cell repairs a double-stranded DNA break in the absence of a repair template. May occur throughout the cell cycle.
Off-target (CRISPR-related): biological activity of the CRISPR-Cas system at unintended sites in the genome. The result can either be destructive or silent.
Postmitotic organ/cell: condition in which a cell (e.g., adult cardiomyocyte) is no longer capable of undergoing mitosis.

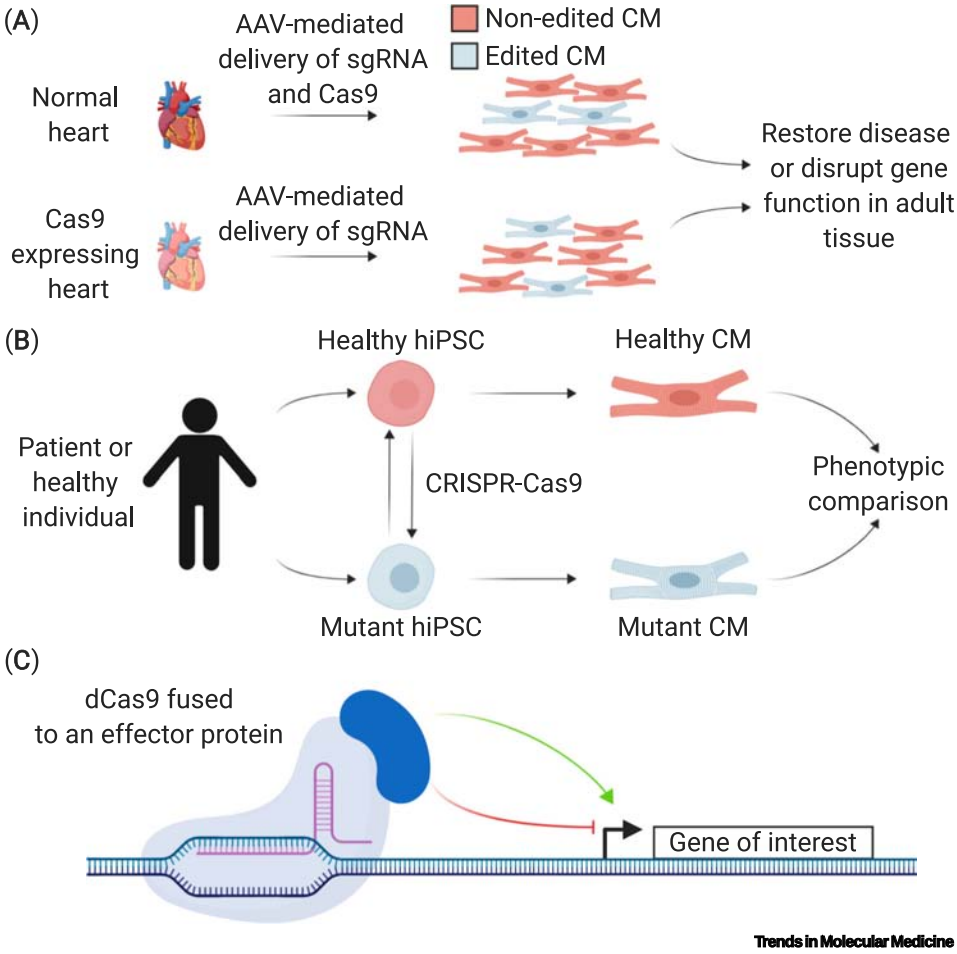

Figure 2. Current Applications of CRISPR in Cardiac Biology and Disease. (A) Cas9 and sgRNAs targeting a gene of interest can be delivered with a cardiotropic AAV construct to the postnatal heart of mice. Alternatively, genome editing of cardiomyocytes can also be achieved by delivery of only a sgRNA to the heart of transgenic mice expressing Cas9. These CRISPR-based methods enable one to interrogate gene function in adult cardiomyocytes, which previously was not always possible due issues regarding embryonic lethality. (B) Biomaterial (e.g., fibroblasts) from healthy individuals or patients diagnosed with a genetic cardiac disorder can be reprogrammed into hiPSCs. Subsequently, CRISPR can be applied to revert the 'disease-driving' variant in mutant cells, yielding an isogenic control. In case patient material is not available, one may utilize CRISPR to knock in the desired variant in healthy hiPSCs. Finally, the mutant line together with the isogenic control can be differentiated into cardiomyocytes, followed by functional phenotyping at desired timepoints. (C) dCas9 fused to transcriptional effector proteins can be used to inhibit or stimulate gene expression of endogenous genes in hiPSC-derived cardiomyocytes. Abbreviations: AAV, adeno-associated virus; CM, cardiomyocyte; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, dead Cas9; hiPSC, human induced pluripotent stem cell; sgRNA, single guide RNA.

may turn out to be critical for efficient targeting. Additionally, several studies have used the smaller Cas9 variant from *Staphylococcus aureus* (SaCas9; 3.2 kb), enabling one to pack one or multiple sgRNAs together with the nuclease in the same AAV vector [21,22]. This single-vector approach assures that a transduced cell receives all required components for genome editing. With respect to Duchenne muscular dystrophy, both strategies are capable of restoring dystrophin levels in the heart with similar efficiency [23]. While these studies show therapeutic potential, future research will have to prove whether these strategies are also applicable to other cardiac indications.

Pronuclear DNA injections: process in which genetic material is injected into the nucleus of a fertilized oocyte. This methodology is often used for the creation of transgenic animals.
Protospacer adjacent motif: short DNA sequence adjacent to the genomic region targeted by CRISPR-Cas. This sequence is essential for proper recognition by the Cas-effector protein and initiates its cleavage activity.
SNP: change of a single nucleotide at a specific position in the genome and should be present in more than 1% of a population. SNPs may drive disease processes.
T-tubules: transverse invaginations of the sarcolemma towards the center of cardiomyocytes. Rich in ion channels, transporters and pumps, hence they fulfill an essential role in cardiac contraction. Well-developed T-tubules are characteristic of mature and functional cardiomyocytes.

To ensure that the presence of Cas9 is not limiting for studying gene function in cardiomyocytes, Carroll and colleagues generated a transgenic mouse model in which the *Cas9* gene was expressed under the control of the cardiomyocyte-specific Myh6 promoter. In a proof-of-principal experiment, the authors used the cardiotropic AAV9 to deliver a sgRNA targeting the *Myh6* locus via a single **intraperitoneal injection** and analyzed the hearts 5–6 weeks later (Figure 2A). They observed robust transduction efficiencies (75%) and a marked decrease in *Myh6* expression, which was accompanied by severe cardiomyopathy and diminished cardiac function [27]. Pu and colleagues used a similar CRISPR-Cas9-based strategy to partially deplete nine different genes specifically in cardiomyocytes to investigate their role in **T-tubule** maturation. In their approach, they delivered an AAV9 construct with cardiac troponin-T promoter-driven Cre and multiple sgRNAs targeting a gene of interest to $Rosa^{Cas9GFP/Cas9GFP}$ neonatal mice. This enabled them to demonstrate that junctophilin-2 is required for T-tubule stabilization in the failing heart, and that ryanodine receptor-2 can function as a novel T-tubule maturation marker [28]. Utilizing the same methodology, this group also demonstrated that a tight regulation of serum response factor activity during the mouse neonatal stages of development is essential for proper cardiomyocyte maturation [29]. Although these results are promising, an additional study showed that these strategies might not work for all targets due to redundancy or **mosaicism** in cellular targeting. This study demonstrated that AAV9-mediated delivery of sgRNAs against *Sav1* and *Tbx20* does not result in a cardiac phenotype, whereas targeting of *Myh6* caused severe cardiac dysfunction [30]. Importantly, these studies demonstrated similar levels of gene disruption for all these targets, underscoring the possibility that certain genes are more sensitive towards genetic modifications than others (see Clinician's Corner).

The use of CRISPR can also be beneficial for generation of mouse models. Traditionally, researchers created knock-in and knockout animal models via **pronuclear DNA injections** or via the injection of modified embryonic stem cells (mESCs) into the blastocyst. However, the cloning strategies exploited to generate targeting constructs or modified mESCs are laborious, ineffective, and expensive [31–33]. By now several reports have demonstrated that delivery of recombinant Cas9 protein together with a sgRNA into the zygote of mice is effective in genome editing [34–38]. Since this implies that a desired mouse model can be obtained within one generation, the time required is reduced to roughly 1 month, instead of several months to a year when standard cloning and targeting methods are applied. However, to the best of our knowledge, so far there are no reports on mouse models designed with CRISPR to study the heart.

While most of the cardiac-related genome editing studies with CRISPR-Cas9 so far have been done in mice, the CRISPR toolbox has also been successfully applied for targeting the genome of other species, such as rats [39–41], rabbits [42], pigs [43,44], zebrafish [45,46], dogs [25], and even, although highly controversial, in human embryos (see Clinician's Corner) [47,48].

In Vitro CRISPR-Cas Applications

Human induced pluripotent stem cells (hiPSCs) have transformed the field of cardiac biology and have provided us with valuable disease models to help define pathogenic mechanisms underlying cardiac disease [49]. A key advantage of hiPSCs is that they can be differentiated into basically any cell type relevant to the heart. CRISPR can be used to correct or introduce disease-driving mutations in hiPSCs to generate a suitable *in vitro* model to study pathogenic mutations with appropriate controls (Figure 2B). The utility of using CRISPR-Cas to generate an *in vitro* model of human heart disease was first shown by Liang and colleagues, who obtained hiPSCs from two patients with type 1 Brugada syndrome (BrS) with two different mutations within the sodium voltage-gated channel α subunit 5. BrS is a channelopathy characterized by an elevated

precordial ST segment, ventricular fibrillation, and sudden cardiac death. This study demonstrated that CRISPR-Cas9-mediated correction of the causative variant restored the disease phenotype back to healthy conditions [50]. In a second study, Ang *et al*. used the CRISPR toolbox to correct a heterozygous missense mutation in the cardiogenic transcription factor GATA4, which is known to cause congenital heart defects [51]. The authors showed that this mutation affected recruitment of TBX5 to cardiac super **enhancers**, which in turn resulted in derepression of noncardiac genes. This resulted in impaired cardiomyocyte contractility, calcium handling, and metabolic activity, which was not observed in the isogenic control [51]. Also related to **hypertrophic cardiomyopathy** (HCM), CRISPR-Cas9 was utilized to better understand the disease mechanism. The authors created multiple hiPSC lines harboring the p.R453C substitution in MYH7, which is one of the most affected proteins in HCM. When comparing the diseased cells to the isogenic controls, they observed all the main hallmarks of HCM, including multinucleation, sarcomeric disarray, and hypertrophy. Furthermore, the mutant cells showed higher metabolic respiration activity, impaired calcium handling, and contraction force. The authors concluded that their findings supported the energy depletion model proposed to be involved in the progression of HCM [52]. In a similar study, Wu and coworkers utilized CRISPR-Cas9 to correct two different *MYBPC3* mutations implicated in HCM. Mutant hiPSC-derived cardiomyocytes displayed the major hallmarks of HCM, which were absent in the isogenic control cells [53]. Finally, Jehuda *et al.* corrected a missense mutation in the *PRKAG2* gene that is involved in HCM. Abnormal firing patterns, delayed after-depolarizations, and structural defects were observed in mutant cardiomyocytes but not in isogenic control cardiomyocytes [54].

Together, these studies demonstrate the value of CRISPR-Cas-mediated reversion or knock-in of mutagenic variants in hiPSC lines, thereby excluding confounding effects due to differences in pluripotency, genetics, sex, and differentiation capacity [55].

Catalytically Dead Cas9 to Modulate Gene Expression

Recently, a catalytically inactive variant of Cas9 (dCas9) has been exploited to modulate gene expression by fusing it to several effector proteins (Figure 2C). For example, Qi and colleagues fused dCas9 to the transcriptional inhibitor Kruppel-associated box (KRAB) and successfully disrupted gene expression in human cells [56]. More recently, Yue and colleagues applied this system to knock down the mutant and wild-type allele of *CALM2* in hiPSC-derived cardiomyocytes of a patient harboring a heterozygous dominant negative mutation (D130G) in this gene. This variant is associated with calmodulinopathy, a disease characterized by severe long QT syndrome due to impaired Ca^{2+}/Calmodulin-dependent inactivation of L-type Ca^{2+} channels [57]. Importantly, CRISPR-mediated knockdown of *CALM2* resulted in restoration of the phenotype.

Besides gene inactivation, dCas9 can also be exploited to enhance gene expression by fusing it to transcriptional activators (Figure 2C). For example, dCas9 was fused to the transcriptional activator domain VP64, which resulted in robust activation of gene expression in human cells [56]. In order to enhance transcriptional activation of endogenous genes, more complex activation systems were developed [58–60]. In one system, robust activation of gene expression was achieved by fusing dCas9 to a tripartite consisting of the VP64, p65, and Rta activator domains [58]. In addition, several groups used optogenetics to modify these activation systems, making them inducible and reversible simply by the use of a laser [61–63]. However, these approaches to enhance gene expression have not been used in cardiac studies.

Current Developments to Improve and Expand the CRISPR Toolbox

While the use of CRISPR has been limited in the cardiac field so far, additional improvements and broadening of its utility will increase enthusiasm for using CRISPR-Cas as a research tool.

Reducing Off-Target Cleavage of CRISPR

An ongoing improvement in the CRISPR field is the reduction in off-target effects. Even though sgRNAs are designed to specifically target a unique genomic region, multiple reports demonstrate that mutations occur at other regions than the desired target site [64–66]. However, the rate of CRISPR-induced **off-target** mutations is unclear, and likely depends on the nature of a particular sgRNA and the biological context in which one wants to modify a desired target [67–73]. This is a major concern for the scientific and medical community as they could unintentionally alter the expression or change the function of unrelated genes.

Recently, a novel methodology termed GUIDE-Seq (genome-wide unbiased identification of DSBs enabled by sequencing) was developed, which makes use of a modified oligo that labels CRISPR-induced DSBs. The authors investigated multiple sgRNAs and compared their computational predicted off-target sites with the CRISPR-induced DSBs observed with GUIDE-Seq. Strikingly, many of the off-targets identified by GUIDE-Seq were not among the expected off-targets, suggesting that the predictive power of these computational programs is inadequate [74].

To increase the reliability and specificity of CRISPR as a genome editing tool, several approaches are currently being tested to reduce the number of off-target events. Shen and colleagues demonstrated that the use of a Cas9 nickase, of which one of the two endonuclease subunits of SpCas9 is inactivated, resulted in efficient on-target cleavage but without detectable off-target effects [75]. In another approach, structure-guided protein engineering was successfully used to increase the specificity of SpCas9 [76]. Kleinstiver and colleagues reasoned that off-target events could be minimized by reducing the number of nonspecific interactions between the SpCas9–sgRNA complex and its DNA target. Indeed, GUIDE-Seq demonstrated a marked reduction in off-target events for the SpCas9–High-Fidelity variant 1 (SpCas9-HF1), which contains four amino acid substitutions (N497A/R661A/Q695A/Q926A) compared to SpCas9. Slaymaker *et al.* developed a different Cas9 variant, termed enhanced specificity SpCas9 [eSpCas9(1.1)], in which the positively charged nontarget strand groove was neutralized [77]. They hypothesized that neutralization of this groove would attenuate the helicase activity of Cas9 when bound to an off-target site due to less-favorable energetics. Unbiased whole-genome off-target analysis and targeted deep sequencing revealed that the eSpCas9(1.1) variant had reduced off-target effects but maintained efficient on-target cleavage activity [77]. In an effort to better understand the mechanism underlying the improved specificity, Chen *et al.* performed a series of biochemical assays, which revealed that the SpCas9-HF1 and eSpCas9(1.1) variants, upon binding to an off-target site, remained trapped in an inactive state, suggesting that the threshold for activation was raised [78]. The authors subsequently identified the REC3 domain of Cas9 as an effector domain that proofreads RNA/DNA heteroduplexes and is required for HNH nuclease domain activation. These structural insights led to the development of a hyperaccurate Cas9 variant (HypaCas9) that contains amino acid substitutions in the REC3 domain. These substitutions improved Cas9 target discrimination and reduced the number of off-target cuts as demonstrated by GUIDE-Seq [78].

Genome Editing in Postmitotic Tissues

Originally genome modifications via the HDR-machinery required DNA replication to allow for the incorporation of exogenous repair templates [79]. As the heart is a **postmitotic** organ, HDR-mediated repair of CRISPR-induced DSBs rarely occurs and might not be relevant [80].

Recently, a novel methodology, termed homology-independent targeted integration (HITI), was developed that supports the integration of exogenous DNA into the genome of nondividing

cells in a HDR-independent manner [81]. This strategy relies on NHEJ-based ligation of CRISPR-Cas9-induced DSBs. By supplying a donor sequence the authors demonstrated robust integration into the targeted genomic locus. In particular, HITI showed a threefold higher knock-in efficiency relative to HDR-based integration events in the neonatal heart.

In another approach, dCas9 was fused to several base pair deaminases. These fusion proteins allow for precise base editing in the absence of a DSB and a repair template, thereby reducing the risk on complex DNA rearrangements [82,83]. Importantly, these base editors act independently from the HDR machinery and may provide a way to edit specific bases in postmitotic cells such as cardiomyocytes (Figure 3A, Key Figure). In an elegant report, dCas9 was fused to APOBEC1, a cytidine deaminase allowing the conversion of a C*G base pair to a T*A base pair [84]. Importantly, this fusion protein only acts on single-stranded DNA, hence the likelihood that random base pair conversions occur is low. One year later, Gaudelli *et al.* used directed evolution to generate an adenosine deaminase fused to dCas9, which is capable of acting on DNA [85]. They demonstrated that this fusion protein achieves high rates of A*T to G*C conversion in human cell lines. Together, the cytidine and adenosine deaminase base editors are able to convert all known base pair conformations, holding great potential for the correction of hereditary cardiac diseases.

A natural occurring variant in the *Pcsk9* gene, observed in African Americans, reduces the risk of coronary heart disease by a striking 88% [86]. Musunuru and coworkers have exploited the base editor system to introduce this variant in mice. They demonstrated efficient conversion of a base pair in the *Pcsk9* gene upon delivery of a vehicle containing dCas9 fused to a cytidine deaminase and a sgRNA to the liver. After conversion, they observed a 50% and 30% reduction in PCSK9 and cholesterol levels in plasma, respectively. Importantly, the researchers did not observe any base editing or indel events at predicted off-target sites as determined by next-generation DNA sequencing [87]. This observation, however, is in disagreement with recent reports that revealed extensive off-target events at the DNA and RNA level for cytidine deaminases fused to dCas9 [88,89]. One explanation for this discrepancy is the difference in detection method used. Zuo *et al.* exploited a novel method termed genome-wide off-target analysis by two-cell embryo injection (GOTI) that allows one to interrogate the off-target effects of a sgRNA in the progeny of genome edited blastomeres [88]. The latter facilitates the detection of off-target events and contrasts other approaches that aim to detect these type of events in mixed cell populations. Importantly, these reports underscore the need for a gold standard for the detection of off-target events. Furthermore, base editors have been the subject of extensive tweaking to improve specificity and efficiency in human cells, which is key in understanding and reducing off-target events [90].

Together, these approaches provide exciting opportunities to modify the genome of noncycling cells, such as cardiomyocytes. Nevertheless, is has to be seen how effective these strategies will be to modify the adult heart. On the contrary, the application *in vitro* should be more straightforward and will likely yield numerous publications in the near future.

Epigenetics and Chromatin Looping

Gene expression is not only influenced by transcriptional activators and repressors but also by the epigenetic state of the cell (Figure 3B,C). DNA methylation and histone modifications are forms of epigenetic modifications that have profound impacts on the epigenetic landscape. In addition, over the years it has also become clear that the 3D chromosome confirmation and density are key aspects in gene regulation, adding yet another layer of complexity. Recently, CRISPR has also been used to induce epigenetic changes and influence chromosomal arrangements; some examples are outlined below.

Key Figure

Future Applications of CRISPR in Cardiac Biology

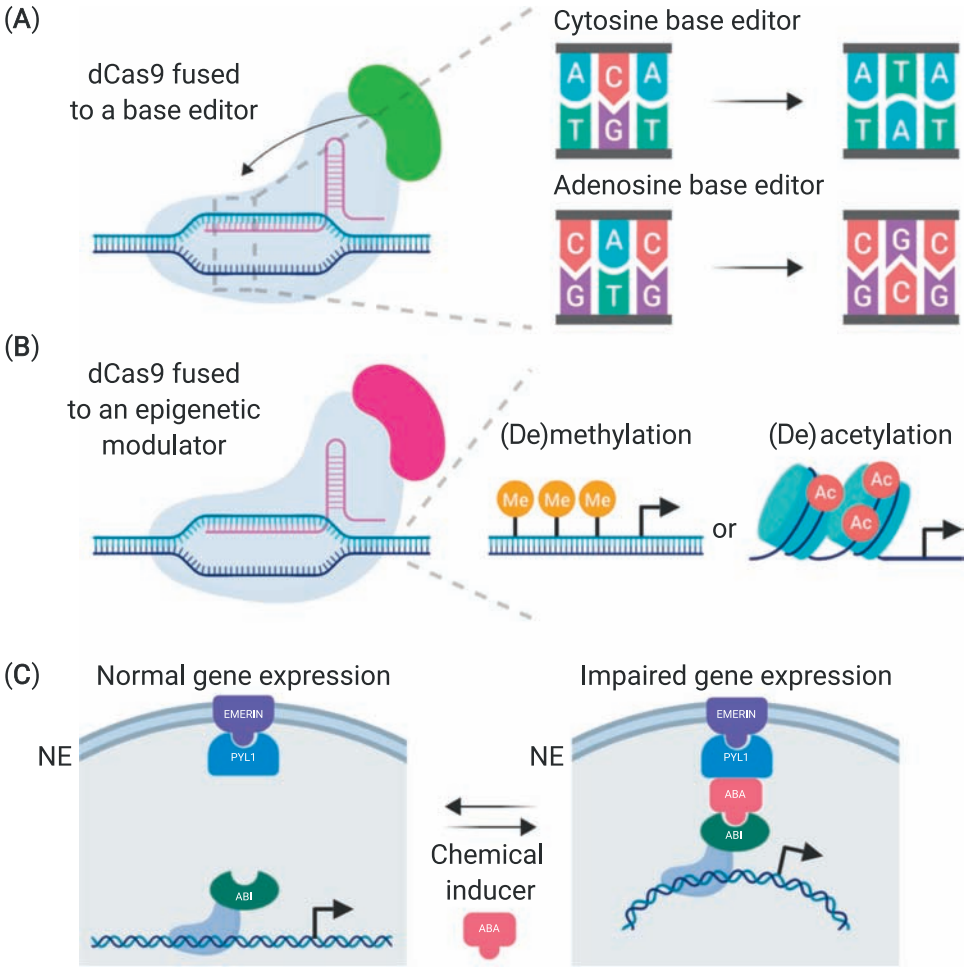

Figure 3. (A) CRISPR base editors provide a means to precisely modify base pairs at any desired genomic region in the absence of a double-strand break. In this strategy, dCas9 is fused to either a cytosine or adenosine deaminase which allows the conversion of a cytosine to thymine or an adenine to a guanine, respectively. (B, C) Novel CRISPR-based approaches provide opportunities to modulate the cardiac epigenetic landscape *in vitro* and *in vivo*. (B) High abundance of methylated cytosines in the promoter region is associated with gene repression, which can be modulated by dCas9 fused to methyltransferases or members of the ten–eleven translocation methylcytosine dioxygenases. Histone acetylation is associated with gene activation, which can be elevated or decreased by fusing dCas9 to histone acetyltransferases or deacetylases, respectively. (C) Genes in close proximity to the nuclear envelope are often repressed. To study the underlying processes, a CRISPR-based strategy can be used to loop desired genomic regions to the nuclear envelope. To achieve this, EMERIN, a protein residing in the NE, is fused to PYL1, whereas dCas9 is fused to ABI. Upon addition of abscisic acid (ABA, chemical inducer), these complexes bind to each other, thereby reorganizing the chromatin landscape. This process can be reversed by removal of ABA from the system. Abbreviations: ABI, ABA insensitive 1; Ac, acetylation; dCas9, dead Cas9; Me, methylation; NE, nuclear envelope; PYL1, pyrabactin resistance (PYR)/PYR1-like protein 1.

Clinician's Corner

Cas9 of *Streptococcus pyogenes* and *Staphylococcus aureus* are the most widely used proteins to modify and modulate cell function. However, it has been shown that the majority of humans harbor a pre-existing anti-Cas9 immunity, which obviously hampers the efficacy of potential CRISPR-based therapeutics [102]. Nonetheless, optimizing the type of delivery vector, dose, administration route and/or utilizing immunosuppressants might aid in minimizing the immunogenicity towards Cas9-expressing cells *in vivo*.

Cardiotropic AAV vectors are the gold standard to deliver CRISPR components to cardiomyocytes *in vivo*. Nevertheless, adult cardiomyocytes are recognized as nondividing cells, hence the viral vector is expressed throughout the lifespan of infected cells due to its episomal nature. Recently, Gersbach and coworkers addressed the long-term effects of AAV-mediated delivery of Cas9 and sgRNAs in a mouse model of Duchenne muscular dystrophy [103]. Even though the expression of Cas9 and the sgRNAs was barely detectable 1 year after administration, sustained expression of dystrophin was observed. Furthermore, the authors revealed a host response to Cas9 and demonstrated that CRISPR-Cas9-induced DSBs may induce AAV integration at unintended genomic loci. To combat these potential adverse effects, non-viral-based methods are currently being developed [104].

At present, CRISPR-mediated genome modification rates in the heart are below 20% [27,28,30]. Depending on the target, these editing rates might be insufficient to alter functional outcome. Alternatively, *ex vivo* modification of patient-derived hiPSCs followed by directed differentiation towards cardiomyocytes yields a homogeneous cell population. Subsequent transplantation of these modified cells into the heart of a patient might therefore be a more effective strategy. However, studies comparing these methods side by side are lacking.

Several groups successfully applied CRISPR to correct disease-causing variants in human embryos, paving

Methylation of the fifth carbon of cytosine (5mC) in the DNA of mammalian cells is considered to be a repressive mark of gene regulation. For example, elevated levels of 5mC are often observed at **CTCF looping sites** and in the promoter regions of repressed genes and vice versa. Jaenisch and coworkers used dCas9 fused to DNA methyl transferase of eukaryotes to methylate a specific CTCF looping site in the genome, thereby preventing CTCF from binding and altering the expression of genes in the neighboring loop [91]. On the contrary, dCas9 fused to the ten–eleven translocation 1 (TET1) protein, a demethylase, is sufficient to induce robust demethylation in promoter, enhancer, and CTCF loci (Figure 3B) [92,93].

Genomic DNA is tightly wrapped around nucleosomes that consist of four histone proteins. These histones, in turn, can be modified by chromatin modifiers, which methylate, acetylate, and ubiquitinate certain residues, thereby altering the epigenetic state of the associated region. Hilton *et al.* utilized the p300 histone acetyltransferase fused to dCas9 as tool to activate gene expression by targeting promoters, proximal and distal enhancers [94]. Furthermore, robust histone demethylation of enhancers was observed when dCas9 was fused to LSD1. With this tool the authors interrogated the functionality of numerous enhancers in embryonic stem cell fate (Figure 3B) [95]. More recently, a second-generation CRISPR-based chromatin-modifier tool, termed FIRE-Cas9, was developed by Crabtree and coworkers [96]. The authors aimed to increase the speed by which chromatin modifiers are recruited to targeted genomic loci. To achieve this, they developed an Fkbp/Frb inducible dimerization system, which consists of two modules. In one module Frb (Fkbp–rapamycin-binding domain of mTor) is fused to a subunit of a chromatin remodeling complex (e.g., Hp1/Suv39h1 or SS18), and the other module comprises the Fkbp (FK506 binding protein) domain fused to a bacteriophage MS2 anchor. Upon addition of rapamycin, these two modules tether to each other and are directed to specific genomic loci by the dCas9–sgRNA complex. Importantly, in contrast to the first generation of CRISPR-based chromatin-modifier tools, this approach recruits endogenous chromatin remodelers. For example, FIRE-Cas9-mediated recruitment of the BAF complex to the Nkx2.9 locus resulted in a significant increase in gene expression just 1 h after addition of rapamycin, compared to several days achieved with the first generation of chromatin-modifier tools [96].

The spatial organization of the genome regulates gene expression. For example, looping of genomic regions to the nuclear periphery is associated with repression of gene expression, whereas looping towards the nuclear interior with transcriptional activation (Figure 3C). However, the relation between looping of specific genomic loci to these compartments is not entirely clear. Wang *et al.* interrogated the function of specific DNA elements by looping them to one of the above-mentioned compartments [97]. To achieve this, the authors developed a novel tool, termed CRISPR-genome organizer (CRISPR-GO), for which they utilized the abscisic acid (ABA)-inducible pyrabactin resistance (PYR)/PYR1-like protein 1 (PYL1) and ABA insensitive 1 (ABI) dimerization system. On the one hand, the ABI domain was fused to dCas9, and on the other hand, the PYL1 domain was fused to GFP-tagged EMERIN, which is a protein residing in the nuclear envelope. Upon addition of ABA, the authors observed a significant increase in reorganization events of targeted regions to the nuclear envelope, which was paralleled by a decrease in gene expression.

Also for the heart, epigenetic regulation and chromosomal (re)arrangements are of importance for development and disease [98–101]. For this reason, it is expected that the above-mentioned CRISPR-based applications for the heart could have far-reaching implications.

the way for so-called designer babies [47,48]. Even though these reports are exciting and hold therapeutic potential, they also bring many ethical dilemmas. Developments concerning CRISPR are actively discussed in society and proper regulation should be in place to avoid misusage [105].

Concluding Remarks

Whether it is the generation of novel (animal) models or the regulation of gene expression by altering the 3D chromosome conformation, CRISPR can do it due to its versatile nature. The simplicity, precision, and efficiency by which CRISPR can modulate all aspects of gene expression are great and will undoubtedly expand even further.

Thus far, the cardiac field has barely touched the extensive possibilities of CRISPR, resulting in only a limited number of publications. These studies mainly applied CRISPR to genetically modify cardiomyocytes or hiPSCs and did not fully explore all opportunities provided by this tool. Its extensive utility guarantees it will gain ground and become a widely used tool for many different reasons.

However, despite all the excitement surrounding CRISPR-Cas, we should remain aware of potential issues, such as off-target effects and ethical concerns (see Outstanding Questions). This issues are becoming more relevant now that CRISPR-Cas is entering the clinical arena as a new therapeutic approach to modulate the genome during disease.

Outstanding Questions

Contradicting reports regarding the rate of off-target effects exist. In addition, most of these studies has been performed in an *in vitro* setting, and therefore it has to be seen how well these results correlate with the *in vivo* situation.

CRISPR-mediated genome editing in adult cardiomyocytes *in vivo* is challenging due to ineffective delivery systems.

Genome editing of cardiac endothelial cells, fibroblasts, and macrophages is challenging due to the heterogeneity of these cell types. Identification of unique biomarkers is key to selective targeting of cardiac subpopulations. If successful, many novel research lines can be initiated.

HDR-mediated repair of CRISPR-induced DSBs rarely occurs in adult cardiomyocytes, thereby hampering the opportunity to precisely modify the genome. Nevertheless, the novel CRISPR-based base editors act in an HDR-independent manner and might therefore provide a solution.

The long-term cellular effects of CRISPR compounds are currently unknown. This is especially relevant when using CRISPR technologies in a clinical setting.

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