Review on CRISPR in Cardiac Repair

By

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Bachelor of Pharmacy (Hons.)

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

The thesis titled "CRISPR in Cardiac Repair" submitted by Tanjia Prodhan Ria, of Summer, 2023, has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

This study does not contain any sort of human or animal trials.

Abstract

This thesis investigates the transformative potential of CRISPR-Cas9 technology in the realm of cardiac repair. Since cardiovascular illnesses are the leading cause of morbidity and mortality globally, innovative strategies are necessary. Acknowledged for its precision in gene editing, CRISPR has potential as a therapeutic approach for cardiac tissue damage and dysfunction. This research delves into the molecular aspects of CRISPR-mediated modifications, emphasising their role in altering significant genes associated with heart regeneration and repair pathways. This study meticulously reviews the literature to shed light on the efficacy and safety of CRISPR therapies in promoting cardiomyocyte regeneration. This paper aims to add to the expanding discourse on therapeutic applications of CRISPR technology, ultimately advancing an understanding of the technology's potential to improve heart repair methods and ultimately reduce the burden of cardiovascular disorders.

Keywords: CRISPR, TLR-4, cardiac repair, PCSK9, gene editing, HDR, NHEJ.

Dedication

Dedicated to my parents for their unwavering support.

Acknowledgement

Firstly, my deepest gratitude to my Almighty Allah for giving me all the strength, patience throughout my journey that has led to fulfilling this work.

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Chapter 1

Introduction

1.1 Background

Global health is gravely threatened by cardiovascular diseases (CVDs), which encompass a broad range of conditions affecting the heart and blood vessels. These ailments include myocardial infarction, coronary artery disease, arrhythmias, and valvular problems in addition to heart failure. Cardiovascular diseases (CVDs) account for a significant fraction of deaths each year and are the primary cause of mortality globally (World Health Organization, 2022). WHO estimates that 17.9 million deaths worldwide are caused by cardiovascular diseases (CVDs), making them the world's top cause of mortality (World Health Organization, 2022). Heart and blood vessel conditions together referred to as CVDs comprise a range of maladies such as rheumatic heart disease, coronary heart disease, and cerebrovascular disease Heart attacks and strokes account for over four of every five CVD fatalities, with one-third of these deaths occurring before the age of 70. Hereditary, ecological, and individual variables interact in a complicated way to cause and exacerbate cardiovascular diseases. Risk factors including inactivity, smoking, diabetes, elevated blood pressure, and hyperlipidemia greatly enhance the likelihood of developing CVDs (World Health Organization, 2022).

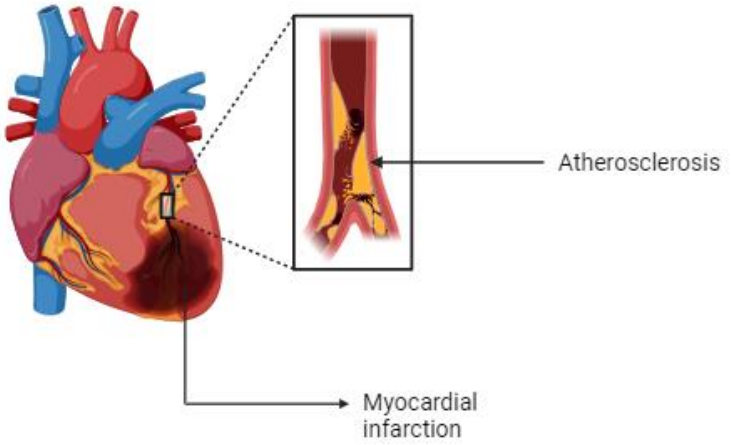

Figure-1: Myocardial infarction, Created with BioRender.com

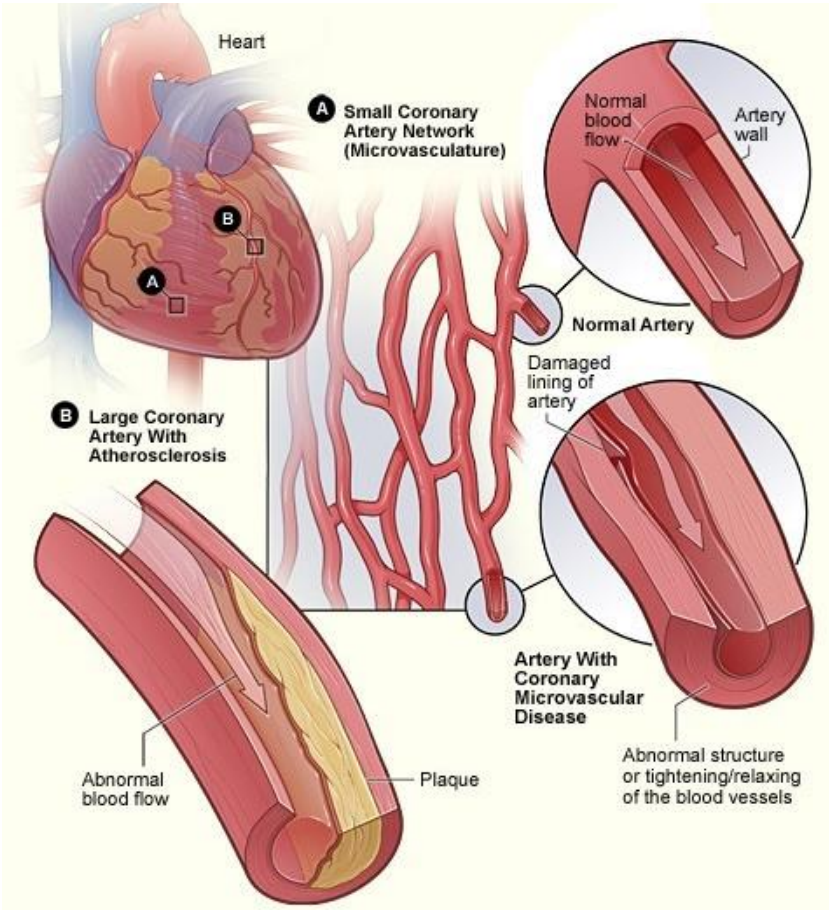

Figure-2: Coronary heart disease, (*Coronary Heart Disease - Causes and Risk Factors | NHLBI, NIH,*)

1.2 CRISPR as gene editing therapy

Cardiovascular diseases are becoming more common as the world's population grows and our lifestyles change, placing strain on healthcare systems and economies. Consequently, a number of technical advancements, such as CRISPR-Cas9, have been made in recent years to aid in the creation of novel therapy approaches for cardiovascular illnesses (Nishiga et al., 2022). Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (CRISPR-Cas9) have fundamentally changed the area of genetic engineering (Nishiga et al., 2022).

With profound implications for biological science, the term "gene editing" implies a broad spectrum of methods for introducing desired alterations at specific genomic loci (Liu et al., 2022). The production of site-specific double-stranded DNA (DSBs) by a few unusual endonucleases in yeast greatly increases the effectiveness of targeted gene integration. As a result, programmable DNA sequencers that may cause site-specific DNA DSB have been developed. The RNA-guided ribosome sequencer (RNA-Cas), which has transformed genetic engineering and genome editing, is one of the four major forms of nucleases (Liu and Olson, 2022).

1.3 Current available treatments

The first gene-editing technique created DNA double-strand breaks (DSBs) at predefined genomic loci using programmable sequence-specific nucleases (SSNs). The DSBs were repaired and the necessary changes were generated with non-homologous end joining (NHEJ), micro homology-mediated end joining (MMEJ), homology-directed repair (HDR) (Liu *et al.*, 2022). The CRISPR/Cas9 gene-editing method requires the Cas9 endonuclease, which breaks double-stranded DNA, as well as template RNA that exactly corresponds with its target gene.

This allows for the rapid, affordable, and simple replacement of genes in cells and animals. (Redman *et al.*, 2016).

A new era of genome editing has been brought about by the creation and use of the CRISPR-Cas system, which has transformed gene targeting and scientific research. The application of genome editing methods is growing, and new CRISPR-Cas systems that improve targeting accuracy and efficacy are being created. Apart from genome editing, CRISPR-Cas has been widely applied in gene regulation, epigenetic modification, and chromatin imaging (Liu and Olson, 2022).

Editing of TLR4 (Schary *et al.*, 2023), iPSC (Zwi *et al.*, 2009), use of conventional and non-conventional tools (Nishiga *et al.*, 2022), prime editing (Musunuru, 2023), base editing (Musunuru, 2023) are some currently available studies in cardiac repair.

Recent years have seen a notable increase in interest in the application of CRISPR-Cas9 in research of cardiac regeneration, which offers revolutionary therapies for heart tissue repair and functional recovery. With this technique, patients can receive customised care based on their genetic profile, modifications to the DNA sequence, and changes to genes involved in tissue healing, angiogenesis, and cell development (Musunuru, 2023).

The present acceptability of genome editing is primarily due to the development of CRISPR/Cas9. Applications-wise, CRISPR/Cas9 offers a simple and effective way to modify genomes both in vivo and in vitro. Because of this, scientists now generally embrace CRISPR/Cas9. Similar to other gene editing techniques, the effectiveness of CRISPR/Cas9 gene editing depends on its capacity to eliminate DNA and on the following activation of repair mechanisms that mend the broken DNA. Certain healing systems have varying degrees of efficacy, particularly in specialised cells. For example, accurate gene editing has frequently

made use of homology-directed repair (HDR), a kind of flawless DNA repair. Only certain times across the cell cycle (S and G2) can lead to HDR (Vermersch, Jouve and Hulot, 2020).

While several DNA repair processes may alter the genomes of adult cardiomyocytes—which do not split—this approach may not be the most appropriate in that situation. The majority of techniques, including HDR, also need the use of a donor template, which makes it more challenging to design and distribute the material to the targeted cells. New donor-independent technologies, including base editors, have overcome these restrictions and increased the possibilities for genome editing (Vermersch, Jouve and Hulot, 2020)

1.4 Limitations

Cas9-induced breaks in the DNA strand initiate the DNA restoration cycle. This approach is required for the inclusion of DNA components such as cDNAs. In addition to the desired gene changes, unintentional alterations such as duplications, unequal or numerous DNA additions, and missing portions are typical. The inability of the repair process to seamlessly incorporate DNA fragments into the genome explains this. Furthermore, if CRISPR/Cas9 fails in embryos, the odds of discovering the target gene are considerably decreased. Because of the large range of gene combinations created by CRISPR/Cas9 technology in mice, identifying unwanted gene modifications is exceedingly tricky (CRISPR Genome Engineering: Advantages and Limitations | Taconic Biosciences).

1.5 Aims of the study

CRISPR is an effective technique with many uses in medicine and research. This work attempts to present a comprehensive overview of the pertinent research so that CRISPR-Cas9 might be used as a revolutionary tool in the pursuit of heart regeneration. It also contains research on the achievements, challenges, and possible future paths to add to the continuing conversation on the use of CRISPR to improve cardiac therapy. CRISPR technology allows for the accurate modification and understanding of genetic data.

1.6 Objective of the study

This study aims to serve as a scientific roadmap, shedding light on the many facets of gene editing research and steering discussions in the direction of possible directions. By means of an exhaustive examination of prior research, it aims to strengthen the groundwork for future research initiatives in this dynamic subject.

Chapter 2

Methodology

A thorough study of the literature produced all the relevant data needed for this investigation. Several trustworthy sources, including specialty websites devoted to a particular topic, journals, online portals with relevant articles and library catalogues, were looked through. The databases listed below were carefully monitored to make sure the most recent data was included:

-Websites run by experts who focus specifically on the topic.

-Extensive journal databases to record a range of scholarly viewpoints.

-Academic libraries' catalogues featuring a variety of materials

-Articles, review papers

A comprehensive search for credible publications, research papers, and review articles was conducted on official websites and databases in order to fully investigate the operation of CRISPR. Data gathering was greatly aided by reliable and well-known sources including PubMed, Google Scholar, Science Direct. Carefully selected keywords like "gene editing," "cardiovascular disease," and "CRISPR tools" were used to locate pertinent studies. The final selection of articles underwent a rigorous screening process to guarantee the highest level of trustworthiness and relevancy. To help with the reference process and maintain the correctness of the credit and recognition of the source works, Mendeley software was used.

Chapter 3

CRISPR tools and treatments

3.1 CRISPR tools comparison

Table 1: Comparison of CRISPR technologies, (Musunuru, 2023)

Technology	Forms of intentional changes	Forms of unintentional changes	Effectiveness	Gene size coding for the editing protein	Elements other than protein editing
Standard nuclease gene editing—non-homologous end-joining	Mild, semi-random indel mutations; precise deletions in the case of two guide RNAs being used	Chromothripsis, or the breaking apart of chromosomes; loss of chromosomal segments; reorganisation of chromosomes; and tiny or large indel mutations	Possibly extremely high (around 100% efficiency).	Moderate (may fit in a single AAV vector in certain situations)	For precise deletion, two guide RNAs serve as a guide.
Standard nuclease gene editing—homology-directed repair	Single-nucleotide changes that are exact, inserts, omissions, and combinations of these	Chromosomal reorganisation, loss of chromosomal regions, chromothripsis (chromosome breaking), and tiny or major indel mutations	Usually minimal	Moderate (in certain cases, might fit in an individual AAV vector)	Repair focused on homology uses guide RNA as a template to repair DNA, either single- or double-stranded.

Technology	Forms of intentional changes	Forms of unintentional changes	Effectiveness	Gene size coding for the editing protein	Elements other than protein editing
Base editing	Single-nucleotide changes (typically restricted to transition mutations)	Bystander single-nucleotide alterations at the target region, RNA and DNA deamination off-target, and minor indel mutations	Possibly extremely high (around 100% efficiency).	Large (often requires splitting over several AAV vectors)	Guide RNA
Prime editing	Exact insertions, deletions, and modifications of a single nucleotide, as well as any combination of these	Indel mutations which are tiny, off-target prime edits, and unidentified effects on reverse transcriptase function	Mostly unfavourable but improving thanks to new technologies	Large (usually necessitates dividing over many AAV vectors)	Twin prime editing or deletion necessitates the use of two prime editing guide RNAs (pegRNAs).

3.2 Standard nuclease gene editing

A few methods (such as the creation of "knockout" mice) permitted gene targeting in animals prior to the latest advancements in gene editing methods. These were difficult, tedious, and highly technical methods. Effective editing of a selected DNA segment is made possible by CRISPR, which transforms gene targeting by generating double-stranded DNA breaks at specific, user-directed places across the genome (Mali *et al.*, 2013).

Around 1990s, the first gene-editing technique was created using nuclease proteins to purposefully cause double-strand breaks (DSBs) targeting certain specific regions within the genome (Musunuru, 2023). The sgRNA spacer series allows the Cas9 protein to the targeted DNA sequences, but only if a brief segment, protospacer-adjacent motif (PAM) trails the target sites. DSBs are generated as a result of the Cas9 protein hybridising to the target locations. To accomplish gene deletion or knock-in, the CRISPR/Cas9 technique needs the endogenous repair mechanisms NHEJ, MMEJ, and HDR (Nishiga *et al.*, 2022).

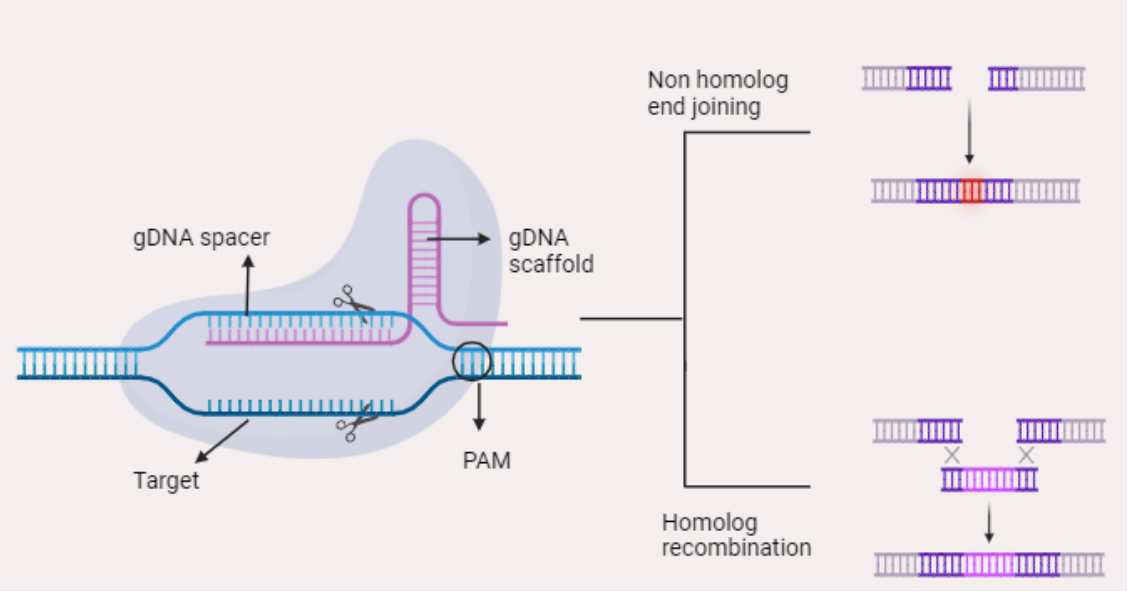

Figure 3: CRISPR basic mechanism, HDR, NHEJ, Created with BioRender.com

Non-homologous end-joining (NHEJ), the most common DNA repair mechanism in cells, is triggered by double strand breaks (DSBs) (Bibikova *et al.*, 2002).

NHEJ may experience indel mutations, or the insertion or deletion of DNA base pairs, as an outcome of the flawed procedure employed to repair the free DNA ends created by DSBs. These indel mutations may cause disruptions to the non-coding regulatory domains (splice sites, in-frame codon additions or eliminations and frameshift mutations) that impact gene expression or the protein products generated by the intended genes. The resulting indel mutations can vary greatly in size, ranging from tiny (a few base pairs) to massive (kilobases).

Because these mutations are semi-random, it is difficult to anticipate with any degree of certainty which mutations will appear in altered cells. Nevertheless, NHEJ has the potential to mutagenesis with a high extent of efficiency—nearly 100% in certain cases. Because of these flaws, NHEJ is ideal for eliminating genes or regulatory elements. Double-strand breaks (DSBs) in the same chromosomal region caused by two nucleases are an appealing strategy for deleting one or more genes. Here, NHEJ will precisely cut off the in-between region and link the ends of the DNA that the DSBs produced (Musunuru, 2023).

The next primary DSB repair technique is homology-directed repair (HDR) (Rouet, Smih and Jasin, 1994). Using a homologous DNA segment as a repair template, homologous DNA repair (HDR) replaces the DNA surrounding the DSB site exactly. This DNA region is present in sister chromosomes, duplicate chromatids on the same chromosome, and synthetic single- or double-strand DNA vectors that have been inserted heterologously. A mutation can be progressively introduced to the genome by HDR if it is constrained by homologous areas in the repair template. HDR is perfect for cassette insertion and introduction or repair of mutations because of this characteristic. HDR is frequently far less effective than NHEJ, even in permissive cells, and NHEJ may make it harder to produce cells with exact genotypes (Musunuru, 2023).

To summarize, both methods begin by cutting the 5′ ends of the DNA break, after which they penetrate a DNA strand that looks like one of the 3′ overhangs. Different sets of NHEJ and HDR repair proteins combine during strand invasion to create a displacement loop, which is further lengthened by DNA synthesis. DSBR pathway-mediated second end ligation and extension cause DNA repair that produces crossover or non-crossover products. On the other hand, non-crossover recombination, or SDSA, is primarily responsible for homologous recombination in mitotic cells (Vermersch, Jouve and Hulot, 2020).

HDR is regarded as error-free since it requires a homologous duplex DNA template in order to do an appropriate repair. The most effective method for precise gene editing and DNA repair is high-dynamic range (HDR), yet HDR has a lot of disadvantages (Liu *et al.*, 2019). To begin with, editing takes time and typically requires selecting the cells that can be modified in a useful way (Nishiga *et al.*, 2022). HDR frequently occurs in somatic cells at lower efficiency rates than NHEJ and is therefore less efficient. This explains why HDR events are so uncommon in mammalian cells. Secondly, the phase of the cell cycle has a major impact on how HDR is implemented (Vermersch, Jouve and Hulot, 2020). Homologous recombination is limited to the S and G2 stages of the cell cycle because it repairs DNA prior to a cell entering mitosis (M phase) (Rothkamm *et al.*, 2003) and thus cannot be applied to cardiomyocytes which are nondividing cells (Nishiga *et al.*, 2022).

Table 2: Difference between NHEJ and HDR repair, (Motta *et al.*, 2017)

Characteristic	**NHEJ repair**	**HDR repair**
Predominant Phase	G1 Phase	S, G2 Phase
Repair Template Requirement	None	Needs a DNA template or homologous chromosome that is not mutant.
DNA Synthesis	Doesn't require an abundance of DNA synthesis	Includes high-fidelity repair by the use of DNA synthesis.
Speed of Repair	Quicker in double-strand break (DSB) repair	Comparatively slow
Indels Introduction	Inserts tiny insertions or deletions (indel) close to the cleavage point	Able to recombinate to produce precise insertions or mutations
Impact on Coding Sequence	Frameshift mutations may occur if an indel is discovered within the coding area.	Allows precise alterations to be introduced without the requirement for frameshifts
Suitability for Gene-Correction	Because of frameshift mutations, gene repair is not appropriate.	Perfect for adding precise mutations without the need for frameshifts
Efficiency in Proliferating Human Cells	Increased frequency and efficiency in the growth of cells	less prevalent and comparatively lower than NHEJ

Even while NHEJ, which is better at repairing DSB, is more likely to occur in human cells in growth, HDR may still be more successful if NHEJ is briefly suppressed (Bozas *et al.*, 2009)

3.3 CRISPR using Cas nuclease-deficient

The two primary functions of the Cas9 proteins in the traditional CRISPR/Cas9 genome editing system are to break double-stranded DNA at the site of binding and to attach to a particular DNA sequence when directed by a sgRNA. A few genomic regions can be targeted without resulting in double-strand breaks when the splitting mechanism is malfunctioning (Dominguez, Lim and Qi, 2016). By inserting mutations into the two nuclease domains of Streptococcus pyogenes Cas9, HNH, and RuvC, this repurposing was first shown (Qi *et al.*, 2013). When directed by a sgRNA, the resultant nuclease-deficient Cas9 (dCas9) may still attach to the desired DNA sequence but cannot break DNA. dCas9 is versatile beyond gene editing since it can bind to specific genes without altering their DNA sequence. Through its interactions with effector proteins, which may alter gene expression patterns or turn genes on or off, dCas9 is able to regulate gene activity. This flexibility makes a wide range of scientific and medical endeavours possible (Dominguez, Lim and Qi, 2016), (Qi *et al.*, 2013).

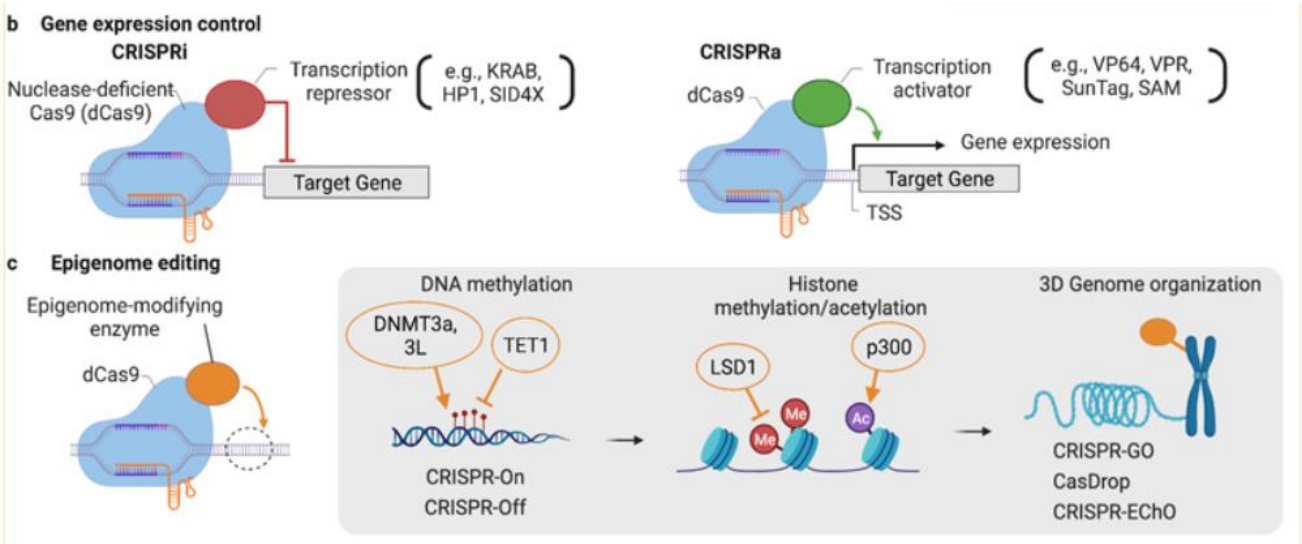

Figure 4: Gene expression control and epigenome editing, (Nishiga et al., 2022)

By altering one of the two Cas9 nuclease domains, Cas9'nickases' and nuclease-deficient dCas9 have been produced (Jinek *et al*., 2012). Instead of producing a double-strand break, Cas9 nickases produce a single-strand break as they possess just one active nuclease domain. Only the intended strand is cut by the RuvC domain-deficient Cas9 D10A nickase. On the other hand, the nontarget (PAM) strand is cut by the Cas9 H840A nickase using an inactivated HNH domain. Based on cas nickases and dependent on nicks, base editors and prime editors are two novel CRISPR tools (Rees and Liu, 2018).

3.4. Non-conventional tools

3.4.1 Base editing

Base editors can be used to install specific point mutations (Kosicki, Tomberg and Bradley, 2018). Base editing allows precise point mutations to be made directly in genomic DNA or cellular RNA without the use of donor DNA, DSBs, or cellular HDR (Rees and Liu, 2018). While the traditional CRISPR/Cas9 process allows point mutations by HDR, there were a number of drawbacks to this approach, such as inadequate functionality, the requirement for blueprints of donor DNA and the reaction of the fixing method of DNA to double-strand breaks, and the inability to use the system in postmitotic cells (Nishiga et al., 2022). DNA base editors are classified into two types: Adenine base editors (ABEs) change A-T into G-C, while cytosine base editors (CBEs) change C-G into T-A. When coupled, they can regulate all four crossover mutations (Rees and Liu, 2018).

Technically, base editors attach to the intended locus and hybridise the sgRNAs to the intended DNA strand to begin the movement of PAM strand. Consequently, an R-loop is created from single-stranded DNA (ssDNA). PAM-distal nucleotides are supplied as ssDNA to the base editors' deaminase region via the R-loop. The cytosines in the R-loop are changed into uracils by cytidine deaminases in CBEs, which polymerases then recognise as thymines. Within the

R-loop, adenosines are converted to inosines by deoxyadenosine deaminases (TadA) in ABEs, which polymerases then interpret as guanines (Nishiga et al., 2022). Specific base pairs in DNA are altered by the DNA base editors adenine base editors (ABEs) and cytosine base editors (CBEs). Whereas ABEs employ synthetic adenosine deaminase domains derived from a single protein, TadA, which is specifically engineered to work on adenine bases in RNA, CBEs use innately occurring cytidine deaminase domains. The diversity of domain sources can be explained by the absence of innately produced adenine deaminase proteins having DNA-binding properties. Because adenine and cytosine base editors may catalyse location-specific single-nucleotide changes, they are commonly referred to as pencil and rubber. At the time of nCas9/gRNA complex attaching to the target genomic region, the deaminase domain linked to nCas9 may communicate with any nucleotides on the non-target strand that fall within a defined range. This access is made clear by the non-target DNA strand adopting a single-strand conformation when the gRNA hybridises with the target DNA strand to form an R-loop structure. Adenine base editors change (A) to (I) and cytosine base editors change (C) to (U). Uracil-DNA glycosylase, a repair enzyme, often changes U to C since DNA is non-standard. But when nCas9, an inhibitor of uracil-DNA glycosylase protein, is coupled with an additional domain, the repair is not possible. Repair to A is arduous, and even if I is similarly non-standard, adenine base modification does not require blocking an endogenous repair pathway. A procedure for fixing nicks that eliminates patch of nucleotide from the target DNA strand is triggered when nCas9's nickase activity is restricted to that specific strand. When a polymerase uses the non-target strand's favourable nucleotides as a blueprint, nucleotide substitution takes place. Whereas cytosine (C) is inserted opposite inosine (I) or guanosine (G), adenine (A) is inserted opposite uracil (U) or thymine (T). T or G is replaced for U or I in the non-target strand after base editing and nick repair, causing a base pair shift (Musunuru, 2023).

Solely A→G and C→T transition mutations may be altered by base editors, and only inside the non-target strand's designated region, which is determined by the PAM position and the combination of base editor and deaminase. There are two more ways to achieve this: either move the base outside of the intended base within the editing window, or make unintentional modifications as a bystander. However, if the targeted change—a nonsense mutation, a mutation at the splice site that deletes a gene, or a mutation correction that causes a disease—can be altered using a base editor, editing can still happen in non-proliferating cells almost 100% of the time. In these cases, base editing combines the remarkable precision of HDR with the remarkable effectiveness of NHEJ to reduce the shortcomings of these conventional nuclease gene editing repair mechanisms. Editing that is off target is still an issue. Off-target editing has been documented predominantly using cytosine base editors, however it is usually restricted to single-nucleotide modifications. Because the deaminase domain interacts with single-stranded DNA regions regardless of the gRNA. Base editing seldom results in large deletions or rearranged chromosomes since it is independent of DSBs (Zuo *et al.*, 2019). Similar to traditional nuclease gene editing, altering the protein can decrease off-target base editing in both nCas9 and the deaminase domain (Musunuru, 2023).

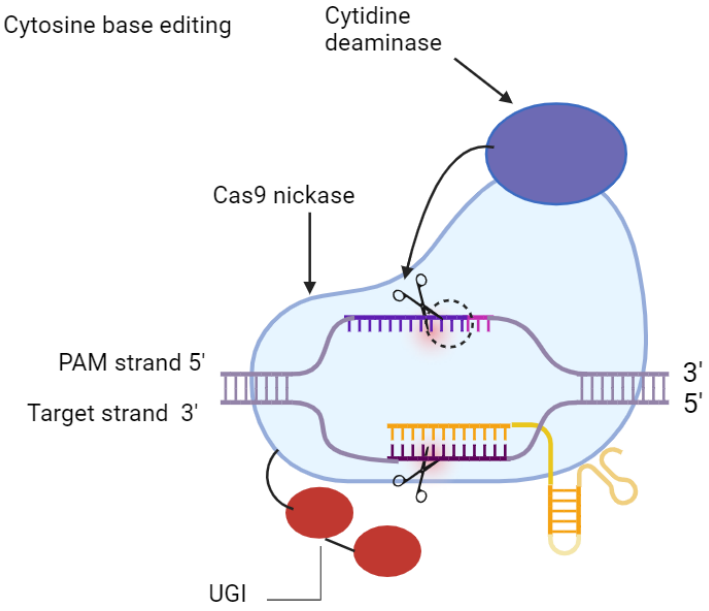

Figure 5: Cytosine base editing, Created with BioRender.com

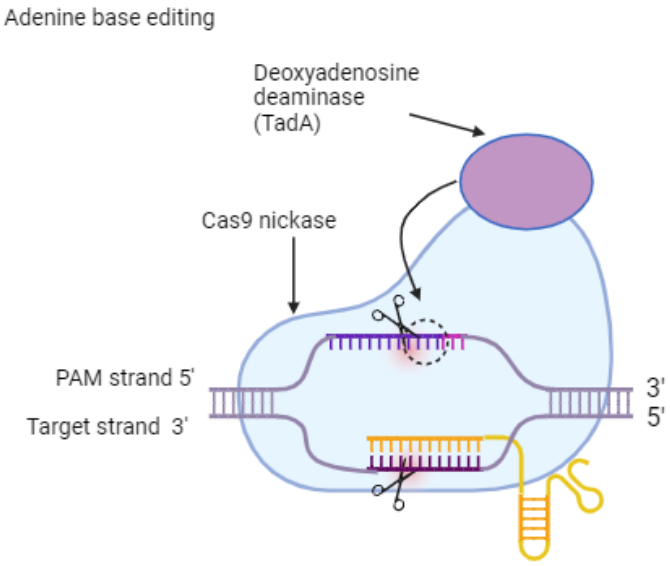

Figure 6: Adenine base editing, Created with BioRender.com

Chapter 4

Clinical trials

4.1 CRISPR editing of TLR4

Mesenchymal stem cells (MSCs) have the ability to create extracellular vesicles (EVs) and free cytokines, which can have effects that are immunomodulatory, reparative, and anti-inflammatory (Schary *et al*., 2023). Nowadays, MSCs are a great substitute for treating heart cells. Because of fibrosis and inflammation, human mesenchymal stromal cells (hMSCs) have limited ability for reparation (Schary *et al*., 2023). According to Schary et al.'s 2023 hypothesis, hMSCs would exhibit a reparative phenotype in the event that the TLR4 gene was disrupted, hence enhancing the effectiveness of cells in repairing infarct. Furthermore, Schary *et al.* provided an efficient electroporation method for CRISPR-Cas9 in 2023. Upon trying on hMSCs isolated from the heart and epicardial fat of ischemic heart disease sufferers, this method demonstrated a 68% success rate (Schary *et al*., 2023).

Table 3: CRISPR editing of TLR4

Name of the study	Study design	Impact of edited hMSC in cardiac remodelling, survival, function after MI				Impact on infarction repair and scar formation			
(Schary *et al*., 2023)	28 days follow up Disrupting TLR4 gene by electroporation Study model- 48 12-week-old female Balb/C mice used in a mouse model of MI	Edited hMSC n=16	**Improvement**	*p* value 0.05	**Comment**	Edited hMSC n=16	**Improvement**	*p* value 0.0009	**Comment**
			90%		A significant amount of improvement is observed since *p* value is <=0.05		90%		A significant amount of improvement is observed since *p* value is <0.05
		Unedited hMSC n=14	80%			Unedited hMSC n=14	75%		
		Saline n=30	70%			Saline n=30	60%		

4.2 Alteration of PCSK9 with CRISPR

Circulation containing LDL-C is one of the most well-known indicators of danger for cardiac disorders, which is the main reason of mortality globally. The most efficient method of lowering the chances of coronary heart disease (CHD) has been demonstrated to be pharmacological therapy that decreases LDL-C levels, particularly statin medications. Even with statin therapy, there remains a considerable residual risk of CHD since many patients are statin intolerant. Thus, creating novel strategies for lowering LDL-C is essential (Ding *et al*., 2014). One possible treatment target to avoid congestive heart failure is proprotein convertase subtilisin/kexin type 9 (PCSK9). First proof pointed to liver-specific gene PCSK9 as the source of autosomal dominant hypercholesterolemia in certain families. Its primary operation is thought to be as an antagonist of the LDL receptor (LDLR) (Ding *et al*., 2014). Gene gain-of-function mutations lead to markedly raised LDL-C levels and early stage of CHD. Those with single loss-of-function mutations in PCSK9 had much lowered LDL-C level (~30%–40%), a decreased risk of coronary heart disease (88%) according to later studies (Ding *et al*., 2014). Remarkably, there appears to be no adverse clinical consequences in individuals with two loss-of-function mutations in PCSK9, that reduce LDL-C levels by over 80% (Zhao *et al.*, 2006).

Given the great efficiency of CRISPR-Cas9 in mammalian cells cultured in vitro (Cong *et al.*, 2013), Ding *et al*. (2014) assessed CRISPR-Cas9's effective disruption of the mouse Pcsk9 gene in vivo and showed that the method may be successful in humans if the gene's ability to target in vivo in mammalian hepatocytes is demonstrated.

Table 4: Alteration of PCSK9 with CRISPR

Name of the study	Study design	Factors	No virus Baseline	GFP virus Baseline	CRISPR Pcsk9 Virus Baseline	End points, *p* value	Comments
Ding *et al*.,2014	Randomized controlled trial 15 female mice of 5 weeks of age	Plasma PCSK9 levels (pg/mL)	21734± 4210	26461 ± 4923	11767 ± 3016	2597 ± 519, 0.0012 vs no virus	Significant PCSK9 levels reduction observed in plasma
		Plasma triglyceride levels (mg/dL)	131.2 ± 15.3	130.8 ± 16.1	129.9 ± 14.2	127.5 ± 14.8 , 0.8765 vs no virus	No significant difference observed
		Plasma cholesterol levels (mg/dL)	161.0 ± 16.2	157.0 ± 15.9	158.9 ± 16.0	101.0 ± 12.3, 0.0001 vs no virus	Significant reduction in plasma cholesterol levels
		Plasma ALT levels (IU/L)	26.3 ± 2.1	25.9 ± 1.9	26.0 ± 2.0	25.7 ± 2.0 , 0.8901 vs virus	No significant difference observed
		LDLR levels (arbitrary units)	100 ± 10	102 ± 11	101 ± 10	120 ± 12 , 0.0009 vs no virus	Significant increase in LDLR levels observed

4.3 Discussion on the clinical trials

There are some fresh breakthroughs in the work of Schary and colleagues. For the first time, they discovered that reducing Toll-like receptor 4 (TLR4) using CRISPR enhanced cell function and the responsiveness to cell therapy in myocardial infarction. They have also made improvements to the method of electroporation, which is used to introduce the altered genes into human cells. Lastly, it has been demonstrated that TLR4 inactivation gives hMSCs from ischemic heart disease patients' reparative and anti-inflammatory qualities. The animals' myocardium, which had experienced a myocardial infarction, underwent infarct healing and scar formation were encouraged by the injection of modified hMSCs.

Yet, there are some restrictions. Initially, the method produced both modified and unedited cells, raising concerns about the impact on 50% of the unedited cells' functional results. Prolonged observation may also highlight notable distinctions between the changed and unaffected cells (Schary *et al*., 2023). Another drawback is the suppression of collagen expression in the conditioned media of the modified human mesenchymal stem cells. Due to the belief that local fibroblasts and hMSCs are the principal collagen makers (Fu *et al.*, 2018), collagen analysis in infarcted hearts was not performed. Implanted cells may directly affect local fibroblasts or by regulating inflammation (Schary *et al*., 2023). A few other limitations are that delayed injections may yield different results that hMSC transplantation may affect the release of DAMPs (Damage-Associated Molecular Patterns), and that immunosuppressive drugs should be avoided because the extent to which MSCs improve heart function is believed to be mainly dictated by the organism's initial immune reaction and any changes that occur to it (Vagnozzi *et al.*,2020), without an immunosuppressant, the altered cells failed to survive for an extended period, despite the fact that inflammation primarily occurred surrounding the unedited cells.

But even after the limitations, the study proposed a new approach that can be proven effective significantly in the limitations are resolved as the study showed potentials.

Ding et al.'s research indicates that the mouse PCSK9 gene was effectively disrupted by CRISPR-Cas9 in vivo. This disturbance was connected to a drop in circulating PCSK9 levels, a rise in hepatic LDLR, and a decrease in plasma cholesterol.

But the CRISPR-Pcsk9 animals had 35–40% lower cholesterol than the control mice, which is consistent with results from prior research that used Pcsk9 knockout mice (Rashid *et al.*, 2005). Following the successful delivery of the CRISPR-Cas9 system to the liver, an adenovirus was used to maintain its expression. An adenovirus-induced immune response may have an influence on plasma cholesterol levels; thus, in order to evaluate any changes, a control group was examined without the virus. Inflammation was not noticeable in the control group. The levels were similar to those of the group infected with the GFP virus. There was no evidence of confounding brought on by the use of adenoviruses over the course of the investigation. One potential issue with this study was no caution for off target mutagenesis was taken into account as this is one of the concerns for CRISPR therapy, although during the clinical trials no proof of off target mutagenesis were seen but low frequency occurrences in in vivo studies cannot be overlooked. Approaches to preserve on-target mutagenesis while drastically reducing off-target mutagenesis are currently being researched upon.

Owing to the wide-ranging effects of cardiovascular illness, human health might greatly benefit from a safe and efficient PCSK9 genome-editing treatment (Ding *et al*., 2014). This study showcased that long-term decrease of LDL-C levels and the threat of coronary heart disease (CHD) may be achieved with a single dosage that provides effects compared to loss-of-function mutations of PCSK9 occurring naturally. In this regard, a single, long-term therapy rather than periodic injections or tablets might signal a paradigm change. CRISPR-Cas9 may be able to

target several therapeutic genes at once with a single treatment due to its multiplexing capacity (Ding *et al*., 2014).

Chapter 5

Advantages of CRISPR therapy

CRISPR allows for quick and adaptable therapeutic design. According to Motta et al. (2017), it is effective at changing various human cells and examining non-coding DNA regions. Single-stranded RNA (sgRNA) or the dual-stranded crRNA-tracrRNA technique are the options available to researchers. The resulting ribonucleoprotein (RNP) complex is formed by the endonuclease and guide RNA joining forces. Editing can occur inside this complex as the guide RNA may link to the target sequence (Thomas, 2023). As long as sgRNAs are delivered and targeted to particular genomic regions, CRISPR-Cas9 may edit several genes simultaneously (Khan, Yuen, and Luo, 2019). For this reason, CRISPR-Cas9 is a compelling and practical technique for modifying distinct genomic areas (Thomas, 2023). CRISPR-Cas9 has the ability to be incredibly economical when compared to other protein-engineering-dependent gene editing techniques like TALENs and ZFNs. With just two molecules needed to make modifications, CRISPR-Cas9 is incredibly simple to employ. Additionally, sgRNAs may be engineered to attach to one or more targets, so researchers might have the ability to start editing rapidly and effectively (Thomas, 2023).

Chapter 6

Limitations

Despite its advantages, CRISPR has disadvantages that must also be taken into consideration. Concerns about the use of CRISPR/Cas9 is mostly related to the remarkably high rate of off-target effects that was noticed occurring ≥50% (Zhang *et al.*,2015). DNA at the seed sequence near the PAM is bound by sgRNA through strict base complementary pairing. Under extreme stress, mismatches may cause the distal three to five bases to take on the structure of an unusual duplex; this defensive mechanism may have evolved in bacteria in response to phage alterations. Whole-genome sequencing and GUIDE-Seq are two techniques for identifying off-target effects (Li *et al.*, 2023). One drawback of the Cas9 method is that a Protospacer Adjacent Motif (PAM) needs to be placed very close to intended area. Because of its often used, short PAM (5′NGG3′), Streptococcus pyogenes Cas9 (SpCas9) presents a challenge when bundled into AAV vectors for gene therapy (Uddin, Rudin and Sen, 2020). DSBs generated by CRISPR frequently result in apoptosis rather than the intended gene alteration (Hu et al., 2014). Like conventional gene therapy, CRISPR/Cas9 has technological drawbacks and raises questions about immunogenic toxicity (Uddin, Rudin and Sen, 2020). In the Charlesworth *et al.* investigation, over 50% of the human subjects acquired anti-Cas9 antibodies against the most often used bacterial orthologs which are SaCas9 and SpCas9. The editing effectiveness of CRISPR/Cas9 technology has also been criticised: The intended target region's sgRNAs are the cause of Cas9-mediated DSB. DSB uses HDR to assist in DNA repair. On the other hand, irregular tiny insertions and deletions might result from a less common appearance of the alternative DNA repair pathway NHEJ (Motta *et al.*, 2017). iPSCs that are created and subsequently given back to patients raise more ethical and practical questions. To truly ascertain the effects of introducing CRISPR/Cas9 into a genetically unique environment and

to appropriately and efficiently transfer the gene editing tool into difficult-to-transfect cell types or tissues, a detailed investigation is required (Motta *et al.*, 2017).

Chapter 7

Future aspects

Comparing CRISPR/Cas9 to other GE mechanisms like ZFNs, TALENs, it is thought to be one of the most helpful mechanisms used in genetic engineering due to high effectiveness, inexpensive price, and the ease of usage of it. The technique of choice for focusing on certain genomic sequences in both simple and complicated species is CRISPR/Cas9, which has an opportunity to significantly alter biological research. While still far from ideal, its target specificity and efficiency have significantly improved. To cut down on errors and clear the path for successful gene delivery and repair, more productive clinical trials are being conducted as well as the evaluation of innovative, non-traditional tools. While there are a number of barriers preventing CRISPR/Cas9 technology from being widely used, new techniques are being developed to increase the technology's efficacy in editing human genes (Manghwar *et al.*, 2019).

Chapter 8

Conclusion

Scientists may now edit faulty genes or remove harmful genes without completely replacing them, thanks to the invention and advancement of CRISPR Cas9. This brings up the possibility of gene therapy once more. CRISPR/Cas9 is a potentially useful technique in modern medicine since it has several potential uses in programmable gene editing (Uddin, Rudin and Sen, 2020). Though CRISPR technology offers great promise for precise genome editing, it is not without limitations. Issues such as restricted vector sizes, off-target effects, and limited PAM sequences highlight the need for more study and development. However, more recent discoveries—such PAMless variations like SpRY—showcase CRISPR's continued advancement and provide fresh opportunities for genomic research and medicinal applications. Since new, unconventional techniques like base editing, prime editing, epigenome editing, and fruitful clinical trials have been developed, it is now possible to study CRISPR and gene editing technology and reduce its dangers and drawbacks. Based on insights gained from traditional gene therapy, more caution has to be exercised in the development of CRISPR systems to prevent unfavourable outcomes and delay the development of a potentially revolutionary and useful treatment. If these lessons are not learned, the development of CRISPR/Cas9 may encounter greater resistance, and the potentially therapeutic genome editing technologies may take longer to develop (Uddin, Rudin and Sen, 2020).

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