Targeting Circular RNA for Cardiovascular Diseases

By

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

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Declaration

Hereby it is proclaimed that

1. The project provided is my own genuine work completed while pursuing a degree at Brac University.
2. No formerly published or written by a third party content is present in the thesis., with the exception of where this is properly cited with complete and precise referencing
3. The thesis contains no material that has been approved or submitted for any other degree or certificate at a university or other institution.
4. All significant sources of support have been acknowledged.

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Approval

The thesis titled “Targeting Circular RNA for Cardiovascular Diseases” submitted by Deepannita Sarkar (18346038), of Spring, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

There are no human or animal trials included in this project.

Abstract

Circular RNAs (CircRNAs) are ubiquitous, and covalently closed loop structures. They are single-stranded molecules without free 3′ and 5 ′ ends. CircRNAs have been rapidly gaining momentum as a potential therapy option for a wide range of cardiovascular disorders, including coronary artery disease (CAD), Cerebrovascular disease (CVD), Peripheral artery disease (PAD), and Aortic atherosclerosis. This review explores several critical aspects such as circRNA biogenesis and suggested biogenesis models, circRNA detection and validation methods, circRNA functions, and current therapeutic uses of circRNA technology. To increase efficacy, these advancements are being researched both independently and in combination with other technologies as well. Significant advances may pave the road for circular RNA technology to be successful in treating these diseases, which are currently affecting a big part of the human population.

Keywords: Cardiovascular diseases (CVDs), Coronary artery disease (CAD), Cerebrovascular disease (CVD), Peripheral artery disease (PAD), Aortic atherosclerosis.

Dedication

Dedicated to my parents

Acknowledgement

I would like to start by giving thanks to the Almighty, who is our inventor and the origin of all life, power, insight, grace, and kindness. All glory to the Almighty who has given me the endurance as well as courage to complete this project. This project would not have been accomplished without the assistance of the individuals who are acknowledged here.

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Chapter 1 Introduction

1.1 Background of Cardiovascular Disease

A group of diseases that affect the heart and blood vessels can be referred to as cardiovascular diseases. Cardiovascular diseases (CVDs) are the prime source of death globally among patients. Approximately, 17.9 million people died from cardiovascular diseases in 2019 that is illustrating 32% of all deaths worldwide(*Cardiovascular Diseases (CVDs)*, n.d.). Generally, it is correlated with a build-up of fatty deposits inside the arteries called atherosclerosis and an increased risk of blood clots. It can also be connected with damage to arteries in organs such as the brain, heart, kidneys, and eyes. Atherosclerosis is a kind of cardiovascular disease that advances when plaque builds up in the walls of the arteries. It became harder for blood to flow through due to this buildup narrowing the arteries. In case of the formation of a blood clot, it can stop the blood flow which can lead to a heart attack or stroke. A broad range of problems can arise within the cardiovascular system; a few of them are rheumatic heart disease, endocarditis, and conduction system abnormalities.

In spite of the fact of cardiovascular disease may directly arise from different etiologies that are emboli in a patient with atrial fibrillation resulting in ischemic stroke, and rheumatic fever causing valvular heart disease, among others, addressing risks factors associated with the development of atherosclerosis is the most important as it is a recognized denominator in the pathophysiology of cardiovascular disease. (Lopez et al., 2021)

1.2 Types of Cardiovascular disease

Cardiovascular disease which is also known as heart disease refers to the following 4 entities which are –

1. **Coronary artery disease (CAD):** It is also referred to as coronary heart disease (CHD). Generally, it results from decreased myocardial perfusion that causes angina, myocardial infarction (MI), and/or heart failure. It is reported that about one-third to one-half of the cases of CVD result from CAD. (Lopez et al., 2021)

   Pathophysiology: The pathophysiology of coronary artery disease is the development of atherosclerotic plaque. Plaque is a build-up of fatty material that narrows the vessel lumen and impedes the blood flow which results from the formation of a "fatty streak." (Shahjehan & Bhutta, 2021)

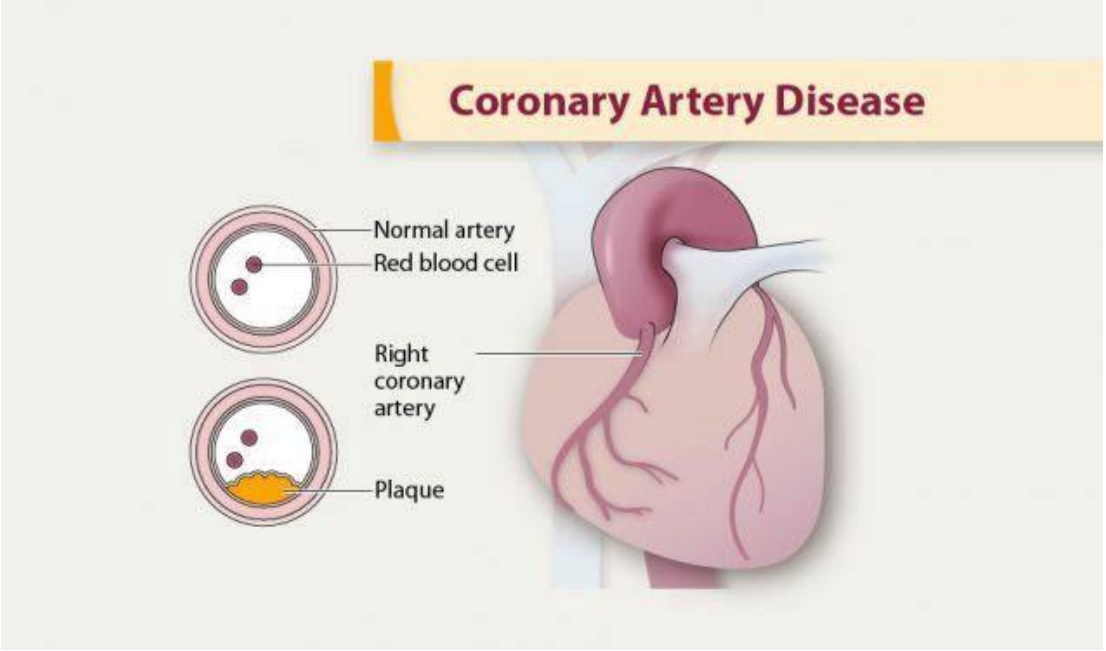

Figure 1: Coronary artery disease (CAD) (Sharma, n.d.)

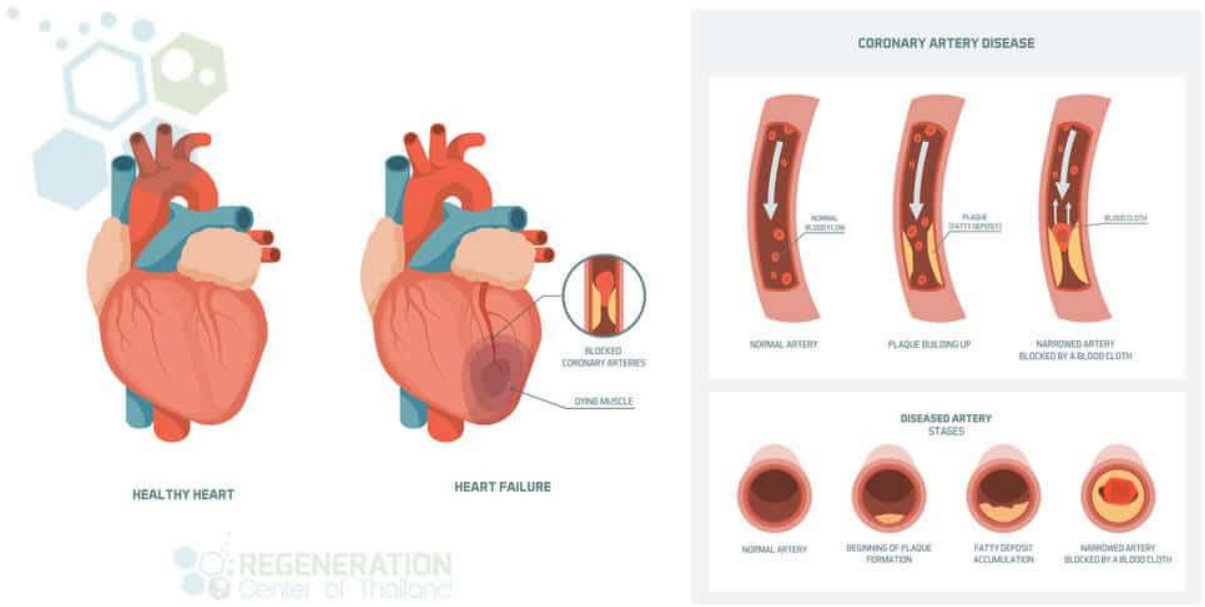

Figure 2: Coronary artery disease (CAD)(Stem Cell Therapy Heart Disease Coronary Atherosclerosis CAD IHD, n.d.)

2. **Cerebrovascular disease (CVD):** Cerebrovascular disease is the entity that is related to strokes which are also called cerebrovascular accidents as well as transient ischemic attacks (TIAs). (Lopez et al., 2021)

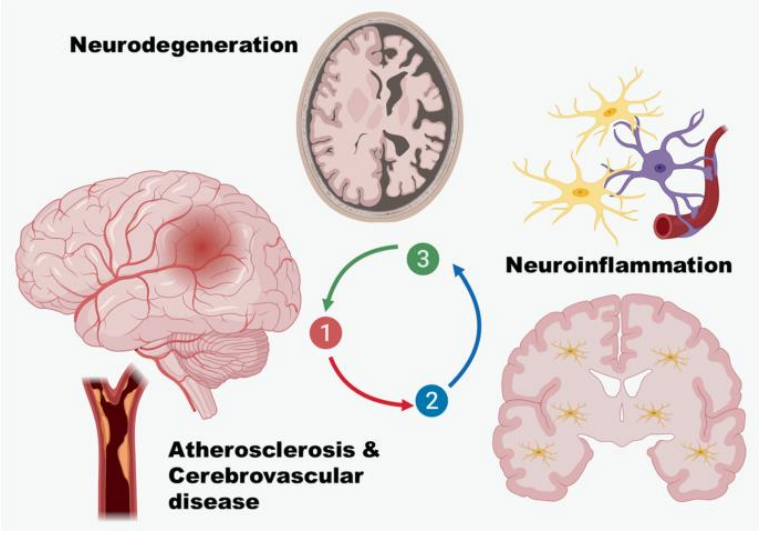

Figure 3: Cerebrovascular disease (CVD)(Toljan, 2022)

Pathophysiology: Ischemic stroke can be caused due to narrowed cerebral arteries. However, tearing vessels on the basis of constantly accelerated blood strain, leads to a hemorrhagic stroke when a reduction in the blood goes with the drift lasting seconds takes place, and the mind tissue suffers insufficient blood delivery or ischemia. (Barpanda, 2021)

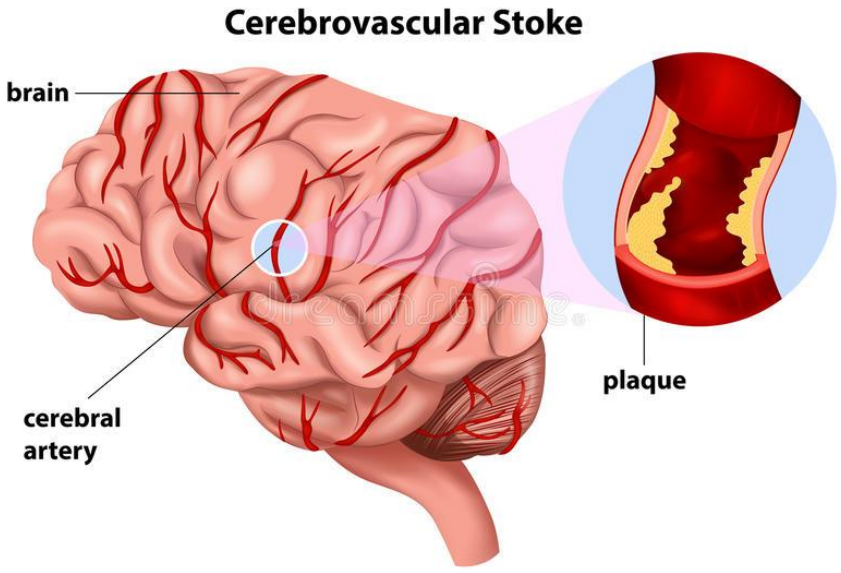

Figure 4: Cerebrovascular stroke(Cerebrovascular Disease Stock Illustrations – 330 Cerebrovascular Disease Stock Illustrations, Vectors & Clipart - Dreamstime, n.d.)

3. **Peripheral artery disease (PAD):** It is an arterial disease predominantly comprising the limbs that may lead to claudication. (Lopez et al., 2021)

   Pathophysiology: Peripheral artery disease (PAD) occurs because of the blockage of the arteries supplying blood to the lower limbs usually secondary to atherosclerosis. The most severe clinical manifestation of PAD is critical limb ischemia (CLI) which is associated with a risk of limb loss and mortality owing to cardiovascular events. (Krishna et al., 2015)

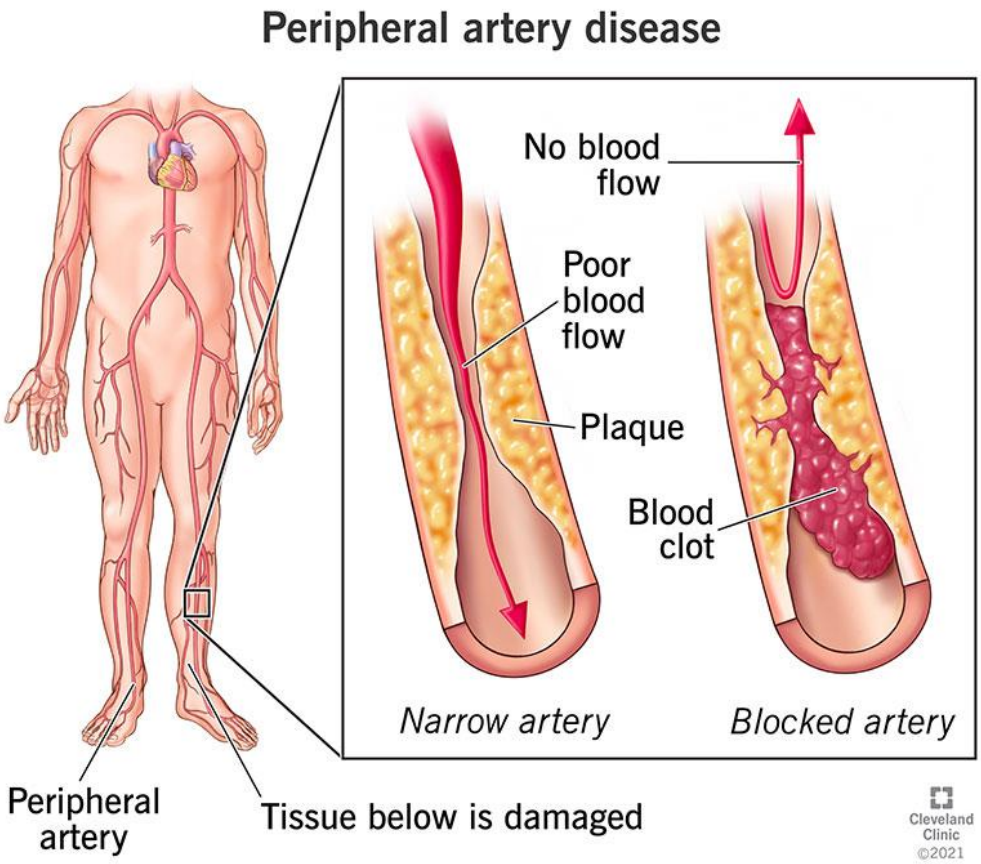

Figure 5: Peripheral artery disease (PAD) (Peripheral Vascular Disease: Causes, Symptoms & Treatment, 2022)

4. **Aortic atherosclerosis:** Aortic atherosclerosis is a cardiovascular disease including thoracic and abdominal aneurysms. (Lopez et al., 2021)

   Pathophysiology: Atherosclerosis is a lengthy process that results in the progressive thickening of the inner layer of the coronary arteries, which can constrict the lumen of the artery to varying degrees over time. Atherosclerosis is a low-grade inflammatory disorder of the intima (inner lining) of medium-sized arteries that is accelerated by risk factors such as diabetes, high cholesterol, high blood pressure, smoking, and heredity. In the case of coronary atherosclerosis, the acute syndrome of the acute myocardial syndrome and SCD

have a preference for the proximal portions of the main coronary arteries, frequently near arterial bifurcation sites that affect the flow in the artery. A cycle of rapid development linked to either of two processes—asymptomatic plaque disintegration with the development of a non-occlusive intraluminal thrombus or plaque hemorrhage—can, however, hinder the gradual progression of atherosclerotic disease. (Ambrose & Singh, 2015)

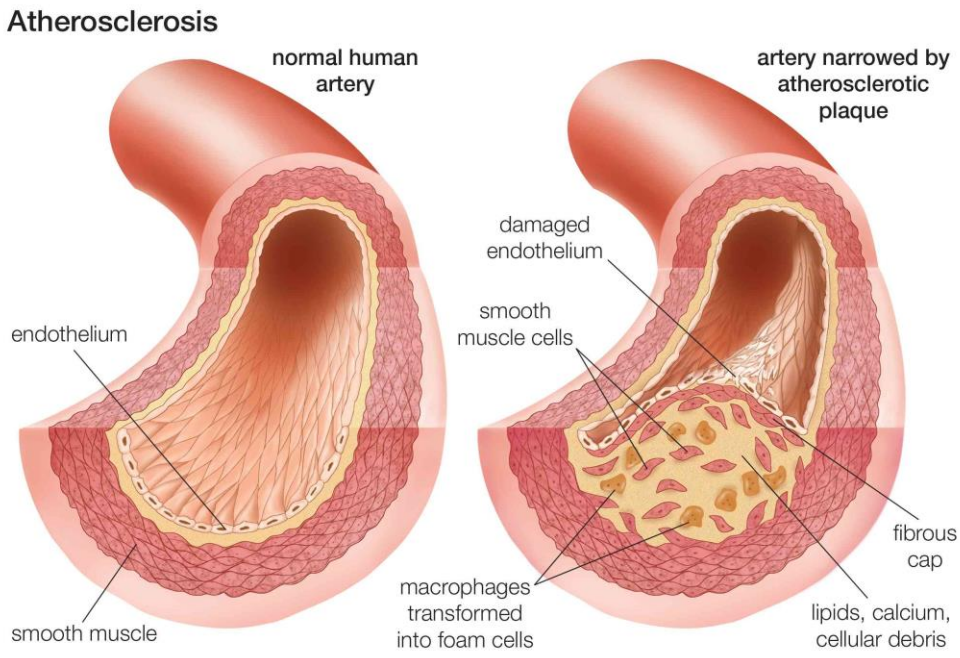

Figure 6: Aortic Atherosclerosis (Atherosclerosis: Definition, Treatment, and Causes, n.d.)

1.3 Current Treatment

From the studies, it has been discovered that cardiovascular diseases are the prime origin of death worldwide (Ghantous et al., 2020).

It is essential to review the morbidity and the impaired outcomes it has on individuals along with its consequences connected to mortality while examining heart disease. Cardiovascular disease is

a wider expression that surrounds disorders of the cardiac muscle, interstitial tissues, or vessels. Additionally, cardiovascular disease can be categorized based on the basic causes, such as rheumatic, ischemia, hypertension, or inflammation; the location of the disease, such as valvular disease, pericarditis, or coronary artery disease; or the type of disease, such as heart rhythm disturbance, infections, or cardiomyopathy. The objective of treating cardiovascular disease is to reduce the number of hospitalizations brought on by cardiac illnesses each year, to lower heart disease-related mortality, and to lower disability caused by heart disease. (Mittal et al., 2018)

Although the most recent treatments for CVD diseases are constantly improving, time is still the most crucial aspect that determines how much dysfunction a cardiac event results in even if the patient survives. Rapid percutaneous coronary intervention (PCI) might enhance the patient's outlook upon discharge. Modern techniques also strive to reduce the time between cardiac episodes and the start of a pertinent medical intervention. Furthermore, there have been a lot of clinical trials conducted recently that aim to lessen or modify the progression of disease as well as preventively reduce the amount of dysfunction that patients experience after an ischemic event. The potential of these approaches to alter the pathophysiology of inflammatory, myopathic, and hypertensive illnesses is also being studied. (Mittal et al., 2018)

At present, in order to treat cardiovascular diseases (CVDs) mainly targeted therapies are pertained such as gene editing technologies, cell therapy, protein drugs, and nucleic acid drugs. (M. Xu & Song, 2021)

1.4 Limitations and prospects

In a nutshell, targeted therapy has become prominent as an innovative and favorable strategy for the treatment of cardiovascular diseases. Additionally, researchers are able to anatomize the pathogenesis of the diseases and survey the targeted therapy by employing proteomics, genomics, and transcriptomics, hence, convoying the treatment of cardiovascular diseases into an accurate therapy period. Regardless, the matter of unanticipated off-target events as well as side effects during the application of the targeted therapies should be confronted in other surveys. Despite targeted therapy has illustrated magnificent usefulness in pre-clinical and clinical trials, various restrictions needed to be acknowledged and conquered in clinical application, for example, gene mutations, off-target events, etc.). (M. Xu & Song, 2021)

1.5 Circular RNA Technology in Cardiovascular Disease

Circular RNAs (CircRNAs) are ubiquitous in all species, from viruses to humans, and are covalently closed loop structures as well as single-stranded without free 3′ and 5 ′ ends. Much progress has been made in the biogenesis, regulation, localization, degradation, and modification of circRNAs. Along with performing as transcriptional regulators, microRNA (miR) sponges, and protein templates, circRNAs influence biological purposes (W. Y. Zhou et al., 2020). The regulatory prospects of circRNAs have still not been entirely discovered though CircRNAs has long been understudied. Originally, circRNAs were concerned with limited isoforms generated as an outcome of binding errors. Nevertheless, it is currently documented that circRNAs are extensively expressed as well as greatly preserved over species with the evolution of bioinformatics and intense output sequencing. The original objective of circRNA was described as acting like a sponge for microRNA to control the production of its target mRNA. Following this discovery, investigations discovered that circRNAs perform a variety of other regulatory

functions, including protein sequestration and translocation, protein interaction facilitation, transcriptional and translational regulation, and protein translation. (He et al., 2021).

Broad-range diseases, typically cardiovascular diseases, cancers, and neurological diseases have been implied in the dysregulation of circRNAs. CircRNAs may be useful therapeutic targets as they are extremely stable and often demonstrate tissue- or cell-type-particular expression. CircRNAs are typically overexpressed employing expression plasmids as well as knocked down through RNA interference (RNAi)-based methods. However, RNAi molecules face several challenges, including limited intracellular penetration, instability, various off-target effects, immune system activation, and a lack of cell-specificity. Though using nanoparticles or exosomes as delivery methods, these RNAi compounds can improve their immunogenicity, intracellular entrance, and stability. The cre-lox technique has recently been used to knock down circRNAs in particular cells. Furthermore, CRISPR technology and Cas13 systems in particular have demonstrated tremendous potential for precisely and robustly knocking out circRNAs. (He et al., 2021)

As a matter of course CircRNAs have lengthy half-lives relative to their linear counterparts, are resistant to exonuclease degradation, and may therefore serve as indicators for illness. Furthermore, recent research suggests that circRNAs may play a significant role in cardiovascular damage and healing. (Kishore et al., 2020)

1.6 Current Circular RNA Technology

CircRNAs, which are quite different from conventional linear RNAs and are found in enormous quantities in the eukaryotic transcriptome, have a closed-loop structure that makes them less

vulnerable to degradation and more stable than mRNAs. CircRNAs typically consist of exonic sequences that are species-conserved and have tissue- and developmental-stage-specific expression patterns. fPractically, circRNAs can serve as miRNA sponges because of their abundance of miRNA binding sites. MiRNAs play a crucial role in conventional advancement and homeostasis *in vivo* as fine-regulators of gene expression, and their dysregulation has been linked to many illnesses. Predominantly, CircRNA plays critical roles via acting like miRNA sponges to bind as well as block miRNAs. CircRNAs can be regulated to adjust the quantity of miRNAs in order to achieve efficacy in monitoring protein levels and biological goals. (X. Zhao et al., 2022)

A wide range of diseases has been circulated with be associated with circRNAs. Because of its stable closed-loop structure and ability to be detected in patient blood and urine, circRNA is presumed to serve as an efficient clinical diagnostic marker in future. Further, this will be efficient for the diagnosis of many diseases as well as contribute to the latest plans for the progress of current targeted therapies and drugs. In spite of the fact that circRNAs-based vaccines or drugs displayed outstanding progress when compared to mRNA, as demonstrated by increased stability, circRNAs drugs are still in the initial stages, with no mature procedures and facilities available for large-scale circRNA generation, which ultimately hinders the progress of circRNAs drugs. Circular RNAs' unique shape allows them to translate and generate proteins of various sizes via rolling-loop translation, as well as translate larger proteins while utilising constrained nucleotide sequences that mRNA drugs cannot. Despite that, the unrestrained collection of antigens or proteins can conduct to unfavorable outcomes. It can be anticipated that various kinds of circRNAs drugs can be progressed in the future with the constant progression of circRNAs technology. (X. Zhao et al., 2022)

1.7 How Can Circular RNA Technology Help Cardiovascular Disease?

In general, despite ongoing research into the causes of cardiovascular disease (CVD) and the advantages of authorized guideline-based therapy, heart failure continues to be the leading cause of mortality globally. High-throughput RNA sequencing (RNA-seq) advancements have made it possible to recognize new transcripts such circular RNAs (circRNA), microRNAs (miRNA), and long noncoding RNAs (lncRNAs). Specific studies have shown that these RNAs regulate biological functions, including the onset and course of illness, which may help in the development of indicative and restorative aids in the favor of the therapy of CVD. CircRNAs are a type referring to short noncoding single-stranded RNAs that are highly conserved between species and are often produced in mammalian tissue. They were discovered by whole RNA high-throughput sequencing libraries reduced of ribosomal RNAs. Additionally, pre-mRNA, from which circRNAs are formed, is back-spliced to produce a molecule with a 3′–5′ phosphodiester bond at the connector location. Numerous circRNAs are produced at reduced levels as a result of back-shortcomings splicing's in comparison to canonical splicing. Exonic circRNAs have also been shown to express themselves at greater levels than their corresponding linear mRNAs in a number of instances. CircRNAs are affluent, evolutionarily preserved, and differently conveyed in cardiovascular illness in a significant way. (Kishore et al., 2020)

1.8 Aims of the Study

This review aims to discover the remaining and latest circular RNA technology in cardiovascular diseases as well as to figure out how circular RNA technology can turn the future of cardiovascular disease treatment.

1.9 Objectives of the Study

The objective of the review is to develop an incisive and informative review that will forward the topic of interest to summarize the remaining cloning study the topic of interest.

Chapter 2 Research Methodology

In order to accumulate all the details and particulars included in this study, a detailed literature review was regulated. The information and evidence came from a variety of reliable references, including online scholarly databases, newspapers, and peer-reviewed journals. The following is a summary of some of the databases that were pursued in detail for the latest study.

- Subject-specific professional websites

- Journal database

- Newspaper database

- Library catalog

In order to gather as much essential information as possible regarding the use of carbon nanotubes in cancer diagnosis and treatment, a thorough search of several journals, review articles and research papers from official websites and research databases was carried out. Utilizing well-known and reliable sources including PubMed, SCOPUS, Google Scholar, and ScienceDirect, the data for this review study was collected. Relevant papers were gathered using appropriate important keywords, such as cardiovascular disease, circular RNA, biogenesis of circular RNA, and use of circular RNA. Articles have been assessed based on the title and keyword content. Then, the papers were reduced after reading the abstracts. The total number of papers that made up this review research were carefully selected and examined. Mendeley software was used for accurate and fair referencing in order to show respect for the writer's original works.

Chapter 3 Delivery of CircRNA Technology

A variety of biological processes, including organogenesis and oncogenesis, depend on circular RNAs as critical regulators. Besides, as RNAs have a role in cardiovascular disorders, they may make good targets for both diagnostics and treatment. Significantly, mounting evidence points to the critical role of circRNAs in organogenesis and pathogenesis as represented by the central nervous system and suggests that they may play a similar role in the cardiovascular system, suggesting a potential therapeutic approach to diseases related to these organogenesis and pathogenesis. (Gong et al., 2019)

3.1. Biogenesis of CircRNAs

CircRNAs, previously thought to be a "splicing error" or "splicing noise," have been rediscovered due to their physiological importance in illness and tissue regeneration. The 3' tail end of the second exon is connected to the 5' head of the first exon to form a covalently closed loop, giving circRNA its unique circular shape. In contrast to canonically spliced linear RNA, which lacked not only the 5' but also 3' termini. Increasing data suggests that these "back spliced" circRNAs are broadly transcribed using a range of spliced isoforms that result in modifications based on the cell type (Salzman et al., 2013). According to RNA-seq data, over 1000 circRNAs have so far been discovered in grownup organs such as the colon, heart, kidney, liver, lung, and stomach. Thirty three circRNAs being highly shared, while between one-third and fifty percent of circRNAs are tissue-specific. Further evidence that fetal cells create much more circRNAs than adult tissues comes from the kinetic variations of circRNAs at different time periods in both mouse and human tissues (Memczak et al., 2013; T. Xu et al., 2017). As a result, circRNAs and their linear counterparts have a dynamic, spatiotemporal specificity (Capel et al., 1993; Cheng et al., 2016; Salzman et al., 2013). Despite the fact of their distinctive circular

form, they are more stable and ideal for use as biomarkers since they have a longer half-life and may be less susceptible to the enzyme ribonuclease R (RNase R). Additionally, they are typically more abundant in expression and more conserved across species, indicating a functional role in life (Enuka et al., 2016). Ever since the first thoroughly researched circRNA, ciRS-7 (CDR1as), demonstrated potential relevance in neurodevelopment, further study on functional circRNAs has been conducted (Memczak et al., 2013). Remarkably, profiling of circRNA revealed the abundance of over 9000 circular species in the hearts of several species, including mice, rats, as well as humans, up to 1288 of which are shared, indicating potential functional relevance for such arrangement (Werfel et al., 2016).

Hypothetical theories presently support the biogenesis of circRNAs as follows:

(1) Direct back splicing: A number of distinct methods contribute to the production of various types of circRNAs. These regulatory elements include RNA binding proteins (RBPs; e.g., Quaking) or spliceosomes by establishing the base pairing-induced tight proximity inside these flanking sequences, which are typically located inside flanking introns (e.g., IRAlus; "intron-pairing") (S. J. Conn et al., 2015; Q. Zheng et al., 2016).

(2) Lariat-driven circularization (sometimes referred to as "exon skipping"): Circularization is promoted by the presence of a "lariat" structure, which is frequently observed by debranching as well as a rapid exonuclease-mediated destruction. This procedure is accelerated by the repetitive complementary flanking components such as the ALU in introns, which generate reverse complementary matches (Vicens & Westhof, 2014; Wilusz, 2015). As a result, circRNAs produced at various genomic loci have diverse structural components and are classed as such. Exon-derived circRNA is the type that has been discovered or investigated the most so far,

primarily for its role in the cytoplasmic transmission of post-transcriptional regulation (Qu et al., 2015). Intronic circRNAs (ciRNAs), on the contrary, is a niche group that is maintained in the nucleus and have an impact on gene transcription (Y. Zhang et al., 2013). Intron-exon circularization also results in the production of exon-intronic circRNA (EIciRNA), something that is thought to somehow be associated in the enhanced parent mRNA transcription (B. Yu & Shan, 2016).

The figure summarizes the biosynthesis and categorization of circRNA and is provided below:

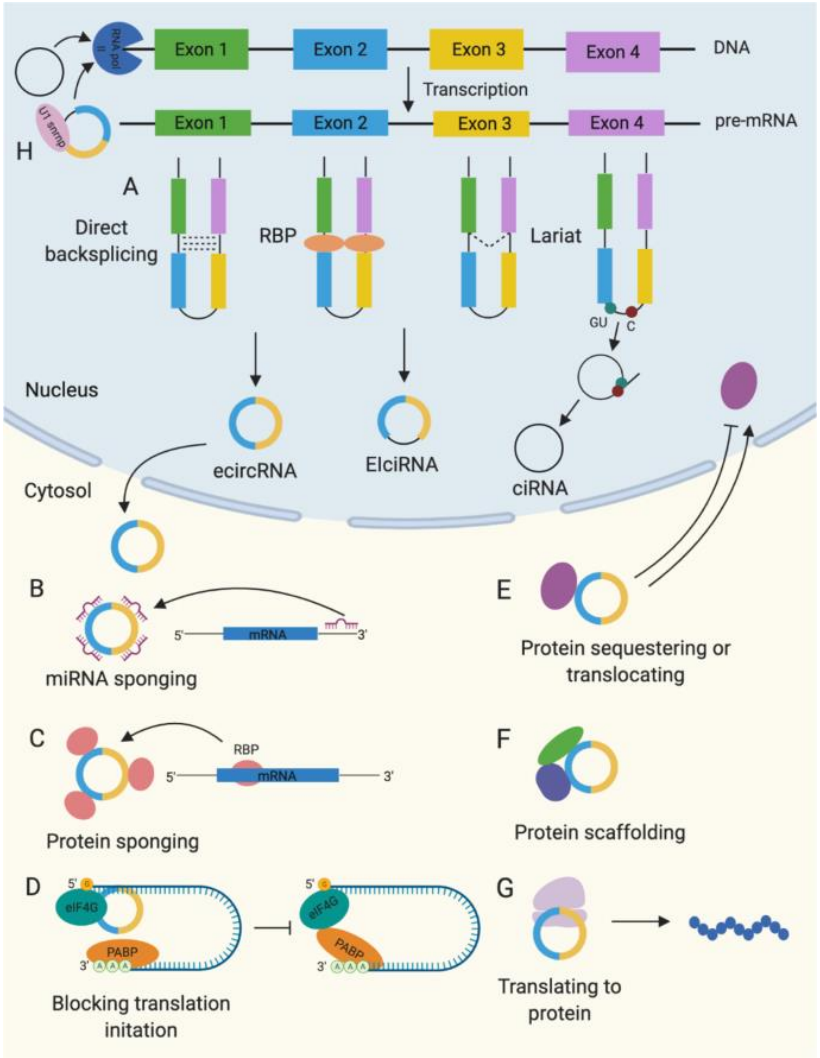

Figure 7: Circular RNAs (circRNAs): Biogenesis and functional mechanisms (He et al., 2021)

A. Back splicing mediated by RNA-binding protein (RBP), intronic complementary sequence pairing, or lariat structures with omitted introns or exons.

B. Sponging microRNA (miRNA) to reduce the amount of target mRNA that they are available to bind.

C. Reducing the availability of RBP to attach target mRNA through sponging

D. preventing the development of the translation initiation machinery by associating with such homologous mRNA, poly(A)-binding protein (PABP), and eukaryotic translation starting factor 4 G or eIF4G.

E. Taking up residence in the cytoplasm or moving proteins to the nucleus.

F. Making certain protein interactions easier.

G. Protein translation that is cap-independent.

H. Exon-intron circRNAs (EIcircRNAs) can assemble with the RNA polymerase II-binding U1 small nuclear ribonucleoprotein (U1 snRNP) to accelerate the transcription of parental genes (RNA pol II). Intragenic circRNAs (ciRNAs) can connect with the stretching RNA pol II complex to enhance transcription. (He et al., 2021)

3.2. CircRNA Detection and Validation Approaches

CircRNA cannot be degraded by RNaseR exoribonuclease because it lacks free terminals, hence enabling RNaseR treatment to enrich circRNAs (Werfel et al., 2016). It is worth noting that some

linear RNAs can resist RNaseR. A research found that RNaseR does not digest more than 20 percent of abundantly expressed linear RNAs (Xiao & Wilusz, 2019). Regarding how to acquire purified circRNA for research of RNA-seq, the RNase R therapy, followed by polyadenylation of linear RNA with free 3′OH ends and associated depletion (RPAD), was suggested (Panda et al., 2017). In a recent study, a strategy to eliminate polyadenylated mRNAs with highly ordered 3′ ends and resistance to RNase R was proposed (Xiao & Wilusz, 2019). use of Escherichia coli for treatment (E.coli) Prior to RNaseR treatment, Poly (A) Polymerase I modified the highly structured RNAs by adding a 3′ terminal poly (A) tail, enabling the enzyme to break them down. RNaseR treatment does not, however, affect mRNAs containing G-rich regions. To resolve this problem, Li+ was added to the buffer solution in place of K+, which is known to support the integrity of G-quadruplex (G4) complexes of polyadenylated mRNA (Xiao & Wilusz, 2019).

Currently, ribosomal RNA reduction and elevated RNA-seq are combined as a detection method. RNA-seq is frequently used to discover novel circRNAs and can reveal details about not only linear RNA but also circRNA. On the other hand, identified back-splicing connectors are the main focus of microarray analysis. In contrast to RNA-seq, microarray study includes recognized circular junction sequence-specific probes to exclusively find identified circRNA (Carrara et al., 2018).

After identification and differential expression analysis, the list of potential circRNAs is verified by RT-qPCR utilizing diverging primers as well as northern blot employing sequence-specific probes that aim the circRNA's back-splicing junctions (Carrara et al., 2018). However, these reverse transcription-based verification methods can potentially result in erroneous circRNA estimations because of template flipping (Kristensen et al., 2019). The proposed pipeline

typically begins with identification using RNA-seq or microarray assessment, is followed by analyses of differential expression selection verification, and finally RNase R degradation resistance testing. As a result, scientists may create databases that combine different data sets to provide information on preservation, expression, and potential efficacy as well as help with primer design. (Kishore et al., 2020)

3.3. Proposed CircRNA Biogenesis Models

By combining exons and cutting out introns from pre-mRNA, the spliceosome mechanism in canonical alternative linear splicing produces a single-stranded RNA with a 5′ cap and poly (A) tail to avoid breakdown (Fig. 8A). Back-splicing procedures rely on canonical splice sites as well as the canonical spliceosome apparatus to generate circulating RNAs (Starke et al., 2015). There are presently four proposed theories that potentially describe circRNA production, while further research into circRNA biogenesis is required. Lariat-driven circularization and intron-pairing-driven circularization were proposed as two models of RNA exon circularization (Jeck et al., 2013). Exon skipping driven by alternative splicing leads to lariat-driven circularization, which can enhance circularization (Fig. 8B). The spliceosome then backsplices the exons, modulating them with both cis and trans regulators (L. L. Chen, 2016). In "intron-pairing-driven-circularization," which promotes circularization, Alu repeats—the most prevalent primate-specific repeats—are placed close together by direct RNA base pairing across introns encompassing exons (Fig. 8C) (Devaux et al., 2017; Diallo et al., 2019; Jeck et al., 2013). An alternate theory for exon circularization proposes that RBP dimerization, which brings exons close together to facilitate circularization, is promoted by bordering introns with RBP binding sites, such as Quaking and Muscleblind (Fig. 8E) (S. J. Conn et al., 2015; Devaux et al., 2017). The RNA splicing factors Muscleblind (Mbl) and Quaking (QKI), members of the RNA family

involved in signal transduction as well as activation, are considered to induce pre-mRNA alternative splicing (Ashwal-Fluss et al., 2014; Darbelli & Richard, 2016). Altogether, research demonstrates that the production of circRNA depends on the presence of intronic binding motifs and the Quaking / Muscleblind binding sites in nearby introns (Ashwal-Fluss et al., 2014; S. J. Conn et al., 2015). Two RNA motif-dependent models for circular intronic (ciRNA) formation have been proposed: the 7-nt GU rich pattern at 5′ splice site as well as an 11-nt C-rich pattern close to the splice site site to promote inefficient de-branching (Fig. 8D) (Y. Zhang et al., 2013). Lastly, diverse exonic circRNAs produced from the same gene locus can develop as a result of competitive RNA pairing across several flanking introns and alternative splicing (Fig. 8F) (L. L. Chen, 2016; X. O. Zhang et al., 2014).

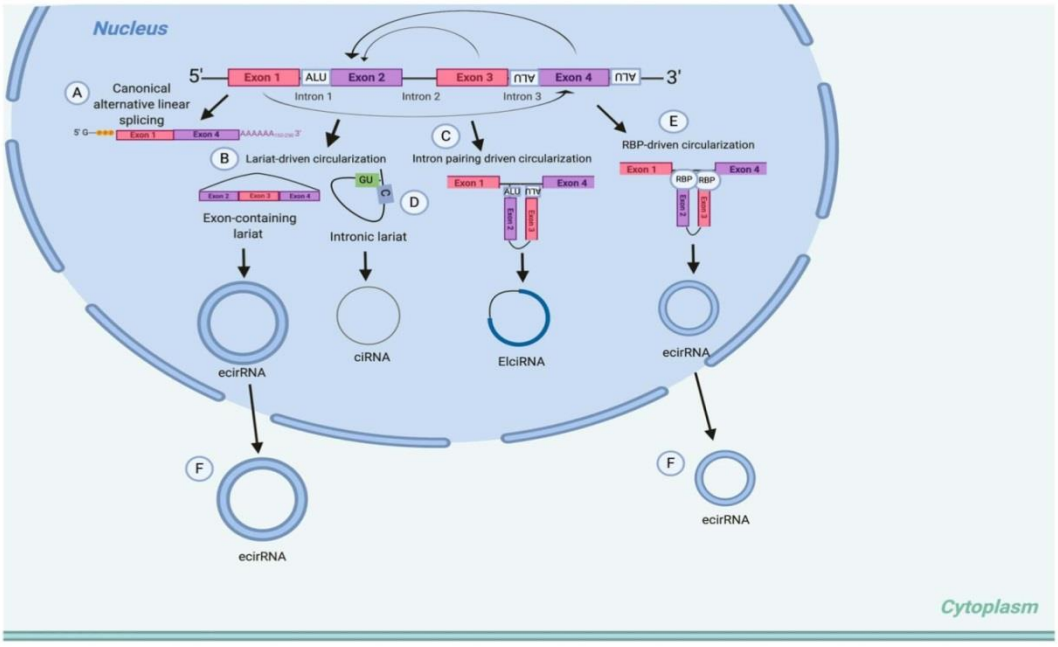

Figure 8: CircRNA biogenesis (Kishore et al., 2020)

(A) Canonical splicing of pre-mRNA. B-D, The spliceosomal machinery facilitates back-splicing circularization to produce various single-stranded covalently closed circRNAs.

(B) Lariat-driven circularization can produce either an intron- or an exon-containing circular RNA.

(C) To enhance the production of exon/intron-containing circRNA, introns with reverse complementary sequences (ALU repeats) are directly paired with circulated exons.

(D) RNA motifs close to the branch point and the 5′ splice sites encourage an intron in order to avoid de-branching and create intronic circRNA.

(E) RBPs can dimerize and bind to bring exons close together to facilitate circularization.

(F) Modified circularization may occur, leading to the production of circRNAs from the same gene locus with variable numbers of exons. Exonic circRNA are generally transported into the cytoplasm after biogenesis, however, circRNA that includes introns stay in the nucleus. (Kishore et al., 2020)

3.3.1. Prevention of Circularization

Double-stranded RNA-specific adenosine deaminase (ADAR) enzymes hinder circularization and prevent the complementation of adjacent introns by replacing adenosine to inosine in endogenous RNA which are double-stranded. ATP-relative RNA helicase A (DHX9) can attach to reversed complementary repeats (Alu) as well as unwind dsRNA helical configurations to prevent intron sequences from looping (Aktaş et al., 2017; Ivanov et al., 2015).

3.3.2. Production of CircRNAs by Co- or Post-Transcriptional Processes

CircRNA synthesis can take place post-transcriptionally or concurrently with transcription. The research on the human genes ZKSCAN1, HIPK3, and EPHB4 discovered that 3′end coding of pre-mRNA ZKSCAN1 is required in favor of the formation of circRNA, as well as short reversed repeats in adjacent introns, indicating that circRNA biogenesis may take place post-

transcriptionally (Liang & Wilusz, 2014). In contrast, it was discovered in a different study that increasing bordering intronic repeats to 260 nucleotides or longer permitted ZKSCAN transcript to generate circRNA without requiring poly (A) signal, indicating that circRNA formation may take place concurrently with other transcription processes (Kramer et al., 2015). Additionally, co-transcriptional circRNA biosynthesis competes with pre-mRNA splicing, resulting in reduced amounts referring to linear analogue (Ashwal-Fluss et al., 2014; L. L. Chen, 2016).

3.3.3. CircRNA and Localization of Characterization

Exonic (EcRNA), intronic (CiRNA), and exon-intron (ElcRNA) circRNAs can be distinguished by their differences in structure, location, and function. The majority of the circRNAs are exonic and are mostly found in the cytoplasm (Capel et al., 1993; Lim et al., 2020; Werfel et al., 2016). CircRNA that is less than 400 nucleotides in length is exported by the ATP-dependent RNA helicase DDX39A. Meanwhile, circRNAs longer than 1200 nucleotides are exported by the spliceosome RNA helicase DDX39B (Kristensen et al., 2019). Intronic circRNA interacts with RNA polymerase II to persist in the nucleus as well as act as modulators of transcription of their corresponding parental genes (Diallo et al., 2019). Current research disclosed the Drosophila helicase Hel25E like a critical nuclear export modulator in favor of circRNAs longer than 800 nucleotides. It is a member of the DExH/D-box protein family, which includes the DEAD, DEAH, and DExH subgroups necessary in the favor of operating of mRNA (Huang et al., 2018; Jankowsky & Jankowsky, 2000).

3.3.4. Circular RNA Packaging into Extracellular Vesicles

Exosomes are microvesicles with a size range of 30 to 100 nm. They contain the prospects to serve as signature molecules in the favor of human disorders and are frequently secreted by various cells to control cell-to-cell transmission (Kishore & Khan, 2016; S. Li et al., 2018). A range of substances, such as nucleic acids, lipids, circRNA, proteins, and microRNA, are present in these microvesicles and they influence the behavior of cells (Fig. 9E) (Kishore & Khan, 2016; Y. Li et al., 2015; Skotland et al., 2017).

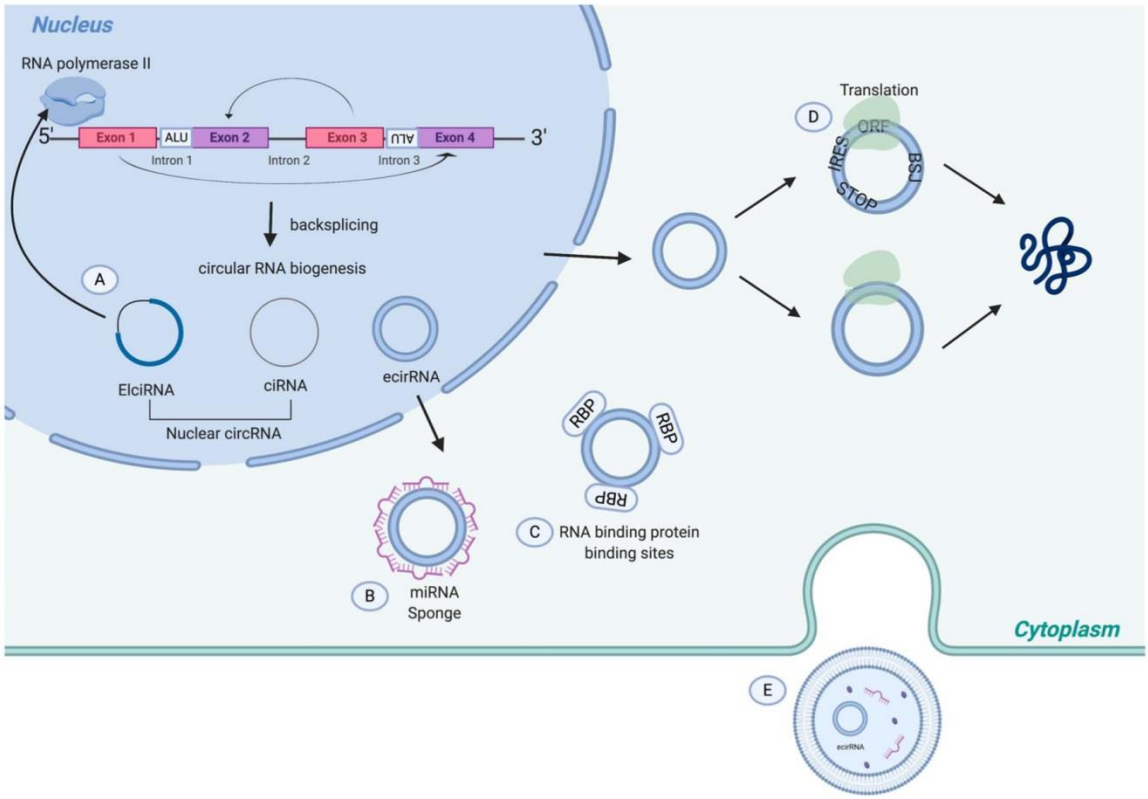

Figure 9: Functions of circRNA(Kishore et al., 2020)

A. A nuclear exon-intron circRNA may control parental gene transcription through binding to the U1 constituent concerning spliceosomal machinery and recruiting RNA polymerase II.

B. CircRNA may bind to miRNAs

C. Interface to RBPs.

D. CircRNA promotes 5′ cap-independent translation by incorporating an IRES element, ORF, or N6-methyladenosine residues.

E. Microvesicles like exosomes can likewise be used to bundle exons carrying circRNA. (Kishore et al., 2020)

Circular RNA (circRNA) may wrapped within exosomes like the means of removing circRNA through the cell since its absence of 5′ and 3′ ends make it resistant to enzyme breakdown and may cause circRNA to accumulate within the cell. Recently, cardiac extracellular vesicles from injured mouse hearts caused by ischemia/reperfusion (I/R) were extracted, and significant differences in the expression of circRNAs were discovered (Ge et al., 2019). Post-I/R damage, there is an acceleration in the discharge concerning cardiac extracellular vesicles. Predicted pathways of circRNAs that are considerably increased include vesicle formation and budding, which is in line with those findings (Ge et al., 2019). These publications emphasize the necessity to look into exosomal circRNAs' potential as a biomarker for CVD. (Kishore et al., 2020)

3.3.5. CircRNA Translation

Multiple circRNAs may be able to code for proteins, according to mass spectrometry and polysome profiling studies of RNase R resistant RNAs. Many studies indicate that circRNAs might have an internal ribosome entry site (IRES) to attract tiny starting components or N6-methyladenosine remnants towards facilitate translation (Fig. 9D) (Diallo et al., 2019; Legnini et al., 2017; Y. Yang et al., 2017). The Muscleblind (mbl) locus is the source of the majority of the circRNAs among the discovered ribo-circRNAs. Fly head immunoprecipitated with Mbl resulted to the production of a 37.04 kDa protein, which was confirmed by northern blot research and

mass spectrometry. Because they have a start codon in common with their linear mRNA equivalent, these described ribo-circRNAs may be involved in regulating the translation of their linear equivalent (Pamudurti et al., 2017).

Other investigations, in the comparison of indicated results, have identified circRNA as noncoding (Guo et al., 2014; Stagsted et al., 2019). They used datasets from the ENCODE consortium for their RNA sequence analysis, and they discovered that AUG circRNAs which are the most widely conveyed and preserved circRNAs. Surprisingly, it was discovered that none of the chosen AUG circRNAs may translated as well as neither mass spectrometry nor ectopic overexpression in cell lines could reveal any peptides (Stagsted et al., 2019). The question of regardless if circRNAs are noncoding or translate within functional peptides/proteins is still being debated. (Kishore et al., 2020)

3.3.6. CircRNA Expression

CircRNAs are widely distributed and interspecies conserved. Their expression depends on the tissue (Salzman et al., 2013) as well as alterations occur as development proceeds (T. Xu et al., 2017; You et al., 2015). Comprehensive RNA-seq investigation concerning rat hearts, human hearts, and cardiomyocytes generated from embryonic stem cells of human revealed that circRNA was tissue-specific, variably expressed, and highly conserved throughout development. However, circRNA expression between patients with cardiomyopathy and those who were healthy did not reveal any appreciable variations in expression (Jeck et al., 2013; Salzman et al., 2012; Tan et al., 2017).

3.3.7. Circular RNA Preservation

Most species in evolution such as metazoans, plants, archaea,yeast, humans, mice as well as rats have circRNAs (Danan et al., 2012; Tan et al., 2017; Werfel et al., 2016). To better understand the circRNA evolution mechanisms and the application, it is crucial to recognize their evolutionary conservation. According to a study, 457 (or 22% of circRNAs) were found in the murine testis and the human fibroblast cell line Hs68. (Jeck et al., 2013), While thirty to forty percentage concerning circRNAs were found in the exons as well as orthologous genes of three different Drosophila species' heads (Westholm et al., 2014). Moreover, half of the observed circular RNAs (9049 of 15,849) shared splice sites in a different research contrasting splice sites of circularization in human as well as mouse encephalons, indicating conservation (Rybak-Wolf et al., 2015). In connection with this research, a one-third splice site conservation rate was found using whole-genome alignment of RNA-seq data from mouse ENCODE cell lines with human orthologs (Guo et al., 2014). It's remarkable to note that only 10% of the circRNAs shared by the hearts of humans, mice, and rats have been shown to be conserved, compared to 30% across mouse and rat hearts (Werfel et al., 2016). For upcoming translational studies, it is crucial to look at highly conserved circRNAs between humans and rodents despite the fact that circRNAs are conserved across species. (Kishore et al., 2020)

Chapter 4 The roles of circRNAs

CircRNAs are involved in a variety of biological processes, including as those that control transcription and translation, sequester miRNA and RBP, and act as disease biomarkers (Fig. 9) (Kishore et al., 2020).

CircRNAs have steadily been discovered to have more particular biological activities, and research continues to be done on how they work. CircNAs have a number of confirmed uses so far (Fig.10).

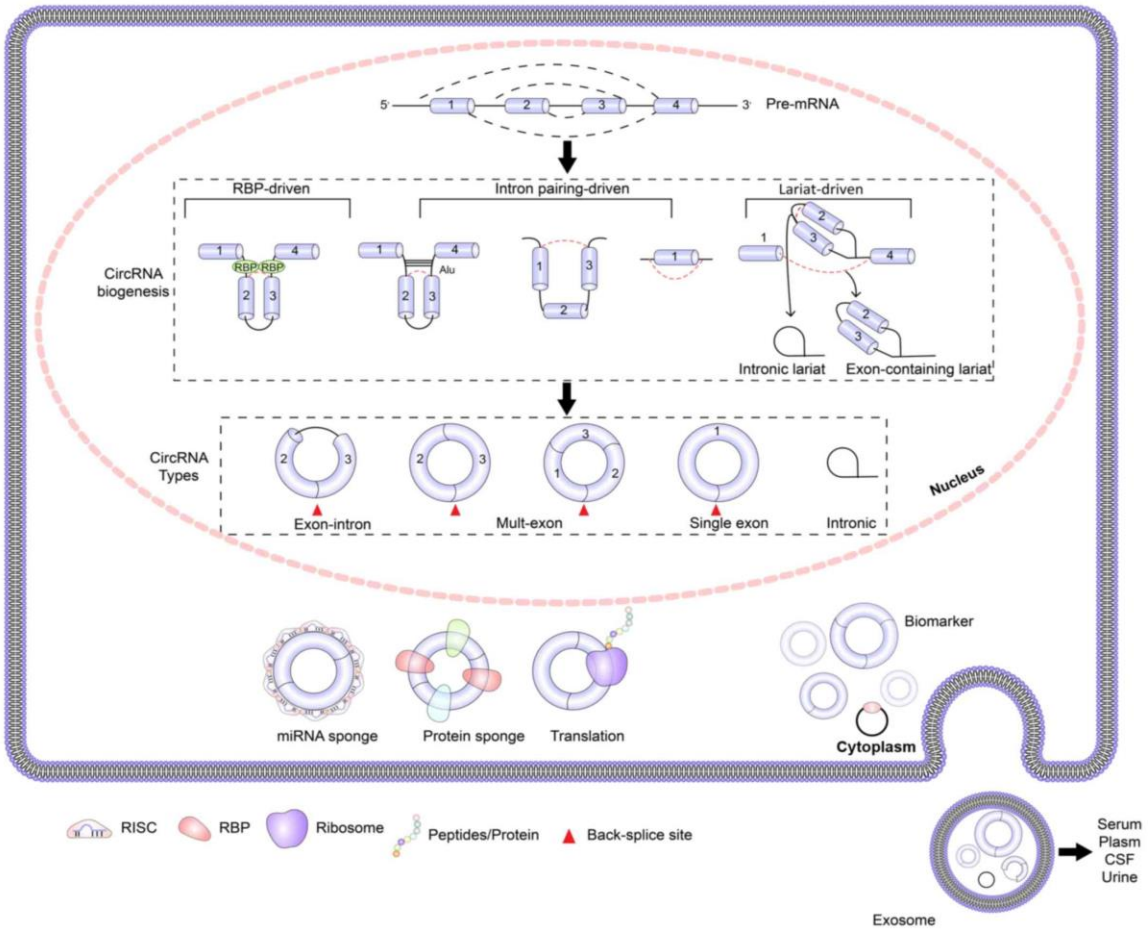

Figure 10: Diagram illustrating the synthesis and application of circRNAs (Z. Yu et al., 2021)

Models for the production of circular RNA include the following: lariat-driven circularization, RBP-driven circularization as well as intron pairing-driven circularization.

CircRNA kinds: exon-intron; intronic single exon; and multiexon are the first two types.

CircRNA performs the following four tasks: translation, protein scaffolding, miRNA sponge, and biomarker. CircRNA is short for circular RNA, while RBP stands for RNA binding protein. MicroRNAs are short for microRNAs, and RISC stands for RNA-induced splicing complex. (Z. Yu et al., 2021)

4.1.1. MicroRNA Sponges

The major part of circRNAs produced via circularizing intron is primarily found in the cytoplasm, with the exception of a tiny number that is found in the nucleus (Kristensen et al., 2019). Numerous studies have demonstrated that circRNAs can engage in binding competition concerning mRNAs in the favor of miRNAs in the cytoplasm, controlling the production referring to mRNAs (Hansen et al., 2013; Memczak et al., 2013). The most representative circRNAs supporting this biological activity are thought to be mouse SRY sponging miR-138 and ciRS-7/cerebellar degeneration-related protein 1 antisense RNA (CDR1as) sponging miR-7 (Memczak et al., 2013). Remarkably, only a limited amount of circRNAs have been found to include various miRNA target binding sites (Guo et al., 2014). Additionally, miRNAs' binding to circRNAs may trigger circRNA degradation (Hansen et al., 2011). It has also been observed that Cyrano, a long non-coding RNA, regulates the CDR1as-miR-7 axis (lncRNA)(Kleaveland et al., 2018). A highly complementary sequence between miR-671 and CDR1as in the non-coding regulatory network causes the rare argonaute2-mediated RNA cleavage that is the target of miRNAs in mammals and even vertebrates (Hansen et al., 2011). According to the

aforementioned results, circRNA-miRNA interconnections both relate miRNA sequestration and exert other worthwhile roles. (Z. Yu et al., 2021)

4.1.2. Competition for Cleavage and Splicing during Transcription with pre-mRNAs

Pre-mRNA atypical splicing produces circRNAs, whereas pre-mRNA conventional linear splicing produces mRNA as the final product (L. L. Chen, 2016). According to a study, increasing the effectiveness of standard linear splicing could significantly lower the number of circRNAs produced (Ashwal-Fluss et al., 2014). Cyclization occurs and normal linear splicing efficiency is greatly reduced when the intron flanking the exon is longer (Liang et al., 2017). These results suggest that pre-mRNA and circRNA can compete for transcriptional attention. (Z. Yu et al., 2021)

4.1.3. CircRNAs act as Protein Sponges and Regulate Transcription and Chromatin Interactions

CircRNAs may attach RNA polymerase II or U1 small nuclear ribonucleoprotein in order to control the parental genes transcription (Z. Li et al., 2015; Y. Zhang et al., 2013). According to a study, when circRNAs are knocked out, the parental genes expression is remarkably reduced (Z. Li et al., 2015). In addition, back-splicing was seen in the RNAs produced by centromeric retrotransposons in maize, and the circular CRM1 RNAs that resulted might connect to the centromeres of the plant through R-loops to encourage the development of chromatin loops. Preceding research has shown QKI inhibited doxorubicin (DOX)-generated cardiotoxicity by attaching to circRNAs produced from the heart's titin (Ttn), formin homology 2 domain

containing 3 (Fhod3), and striatin 3 (Strn3) (Gupta et al., 2018). Additionally, circRNAs have binding sites for the host's RBPs and can control how much of them are expressed. For instance, circRNA zinc finger protein 609 (ZNF609) can control the quantity of p-protein kinase B (Akt) and the ratio of phosphorylated (p)-Rb/Rb, hence influencing the advancement of the G1/S phase in cells (Legnini et al., 2017).

4.1.4. CircRNAs Regulate Transcription and Translation

Exon-intron circRNAs (EIciRNAs) in the nucleus control how the parental genes are expressed. EIciRNAs work as a positive feedback loop by interacting with the spliceosomal machinery's U1 component to attracting RNA polymerase II to the parental gene's promoter site to increase expression (Fig. 9A) (Z. Li et al., 2015).

CircRNAs can also influence the translation of their linear equivalent wheather the ORF spans the linear transcript's start codon (Legnini et al., 2017; Pamudurti et al., 2017). CircRNA can interact with RBPs to control translation (Fig. 9C). CircPABPN1 which is the most abundant HuR targets among the discovered circRNAs, and it competitively inhibits PABPN1 mRNA translation by suppressing HuR binding to PABPN1 mRNA (Abdelmohsen et al., 2017). The isoproterenol-induced remodeling of the myocardium was accelerated by the ablation of HuR in cardiomyocytes (Hu et al., 2020). HuR's role in cardiac remodeling and heart development has been extensively studied, although it is still unclear if HuR-circRNA complexes contribute to CVD (Kishore et al., 2020).

4.1.5. miRNA or RBP Sequence-estering via CircRNA

CircRNA's most well-known use is as miRNA or RBP sequesters to control mRNA transcription, processing, and stability (Figs. 9B, 9C). CDR1as is a well-known circRNA that can behave like miRNA sponge and is recognized to sequester miR-7 in the brain of mice (Hansen et al., 2013).

Although absence of an affluent of attaching regions for certain miRNAs, the majority of circRNAs nevertheless control miRNAs (Fan et al., 2017; Haque & Harries, 2017). Recent research indicates that some circRNAs might not function as miRNA sponges (Garikipati et al., 2019; Guo et al., 2014; Militello et al., 2017; Stagsted et al., 2019). No statistical significance was found while looking in the favor of circRNA affluent with argonaute (AGO)-enclosed areas, a protein that is a component of the RNA-generated silencing complex and is recognized toward directly attach with miRNA (Militello et al., 2017). Furthermore, stoichiometric evaluation of liver and hepatocyte miRNA target locations (Denzler et al., 2014), Experiments on human umbilical vein endothelial cells that have lost their function (Boeckel et al., 2015) as well as in silico research on projected 8mer target locations in many species (Stagsted et al., 2019) bolster the idea that circRNA might not control miRNAs. Recent research from our group found that mutations of the anticipated miRNA binding sites for circFndc3b had no impact on the circRNA's ability to operate both *in vitro* and *in vivo* (Garikipati et al., 2019). It is unknown if circRNAs without miRNA/RBP binding sites can not directly control them or play various part entirely, even though the majority of discovered circRNAs serve as miRNA or RBP sequesters. (Kishore et al., 2020)

Chapter 5 Current Clinical Applications of CircRNA Technology

Through an RNase R enrichment strategy, 575 circRNAs in the grownup murine heart were discovered in 2016 (Jakobi et al., 2016). It was subsequently discovered that a lot of the previously mentioned circRNAs were generated through loci of gene linked to CVD, illuminating the roles of cardiac circRNAs in disease. Then it was discovered that the hearts of mice, rats, and humans each had more than 9,000 circRNAs. Rat and mice share about 30% of the same genes, and the three species share 10% of the same genes (Werfel et al., 2016). Additionally, the authors discovered that circRNAs preferred to contained in the cytoplasm of newborn rat cardiomyocytes (NRCMs) (Werfel et al., 2016). Additionally, by using RNA sequencing with ribosomal depletion, Finally, it was discovered that humans and mice, respectively, had 15,318 and 3,017 circRNAs and that the affluence of the indicated circRNAs was often associated certainly with the analogous linear RNAs (Tan et al., 2017). CircRNAs play expanding significant implications in CVDs in addition to physiological circumstances. The circRNAs and their involvement in various CVDs have now been concisely outlined (Table 1) (Liu et al., 2021).

Table 1 circRNAs in cardiovascular diseases

circRNA	Species	CVDs	Functions
Cdr1as	Human/ mouse	MI	Aggravates MI injury through *Cdr1as*-miR-7a-PARP/SP1 axis
circ-Amotl1	Human	MI	Exerts a protective effect on MI through promoting AKT1 phosphorylation and nuclear localization
HRCR	Mouse	HF	Serves as the sponge of miR-223 to increase the expression of ARC, and then inhibiting cardiac hypertrophy and HF
circANRIL	Human	AS	Promotes apoptosis and inhibiting proliferation in VSMCs and macrophages through inducing p53 activation, and thus conferring a protective effect on AS
circWDR77	Human	AS	Promotes proliferation and migration of VSMCs and the progression of AS through *circWDR77*/miR-124/FGF-2 axis
circ-Foxo3	Human/ mouse	Cardiac senescence	Serves as the protein sponge of senescence-associated proteins to prevents these proteins entering the nucleus, thereby leading to heart senescence
circ_0005870	Human	HT	Might serve as the sponge of several miRNAs to exert effects in HT
circ_0037911	Human	HT	Could be a stable biomarker for early diagnosis of EH
circRNA_000203	Mouse	Cardiac fibrosis	Serves as miR-26b-5p sponge to promote the expression of Col1a2 and CTGF, thus accelerating the proliferation of cardiac fibroblasts
circRNA_010567	Mouse	Cardiac fibrosis	Accelerates cardiac fibrosis through *circRNA_010567*/miR-141/TGF-β1 axis

Figure 11: CircRNAs in Cardiovascular Diseases (Liu et al., 2021)

5.1 CircRNA-based Cardiovascular Disease Therapeutic Strategies

Depending on the specific type and severity of CVDs, current therapeutic approaches range from pharmaceutical therapies and lifestyle changes to surgery and transplant. Despite improvements in treatment methods, CVD mortality is still high (Bonsu et al., 2016). The delivery of siRNA, miRNA, or plasmids into the body via adenovirus, retrovirus, or other means is referred as gene therapy. CircRNA-related gene therapy can a potential method to medicate CVDs given the roles that circRNAs play in these diseases and their unique qualities. In contrast, for those circRNAs that may encourage the incidence or CVDs, shRNA, siRNA advancement or function-related techniques waste might be used to alleviate associated CVDs. This is true for circRNAs that play protective roles in CVDs.(Liu et al., 2021)

Several circRNA-based treatment plans for CVDs based on biological or material components have recently been outlined (Table 2).

Table 2 Summary of circRNA-based therapeutic strategies for CVDs

Therapeutic strategies	Advantages	Disadvantages
Transient transfection strategy	Availability due to easy synthesis; time saving	The effects lasting for a short time; triggering an innate immune response; potential off-target effects; Unspecific organ or tissue expression
Viral vector-based strategy	High transduction efficiency, stability, and tissue- or organ-specificity; low pathogenicity	Activation of innate immune; genomic integration
EV- or exosome-based strategy	Resistant to degradation; rapid cell take-up via specific surface markers	Unknown optimal dose, route of administration, etc.
Nanoparticle-based strategy	Improved drug delivery to inaccessible intracellular targets; tissue-specific or cell-specific delivery	The lack of popularity of nanotechnology

EV, extracellular vesicle.

Figure 12: An overview of circRNA-based treatment approaches for cardiovascular diseases (Liu et al., 2021)

5.1.1. Transient siRNA or Overexpression Vector *In Vivo* Transfection

Intraperitoneal injection of the circ-Foxo3 plasmid generated senescence as well as exacerbated doxorubicin-influenced cardiomyopathy, according to a study. Circ-Foxo3-targeted siRNA intraperitoneal injection blocked senescence which alleviated cardiomyopathy, as expected (Du et al., 2016). Furthermore, a study demonstrated Cdr1as overexpression *in vivo* through pcDNA-Cdr1as vector intracardial injection in a mouse myocardial infraction injury model (Geng et al., 2016). These findings imply that uninterrupted siRNA or delivery of overexpression vector circRNA *in vivo* could be a productive CVD therapeutic strategy (Figure 11). Eventhough its

time savings as well as availability, the results in transient transfection may only be temporary. On the one hand, and the initiation of exogenous DNA or RNA molecules may stimulate an innate immune response on the other. A lot of chemical regulation like phosphorothioate, cholesterol, and phosphate, should enhance siRNA anti-ribonuclease activity as well as adsorption of cell. (Liu et al., 2021)

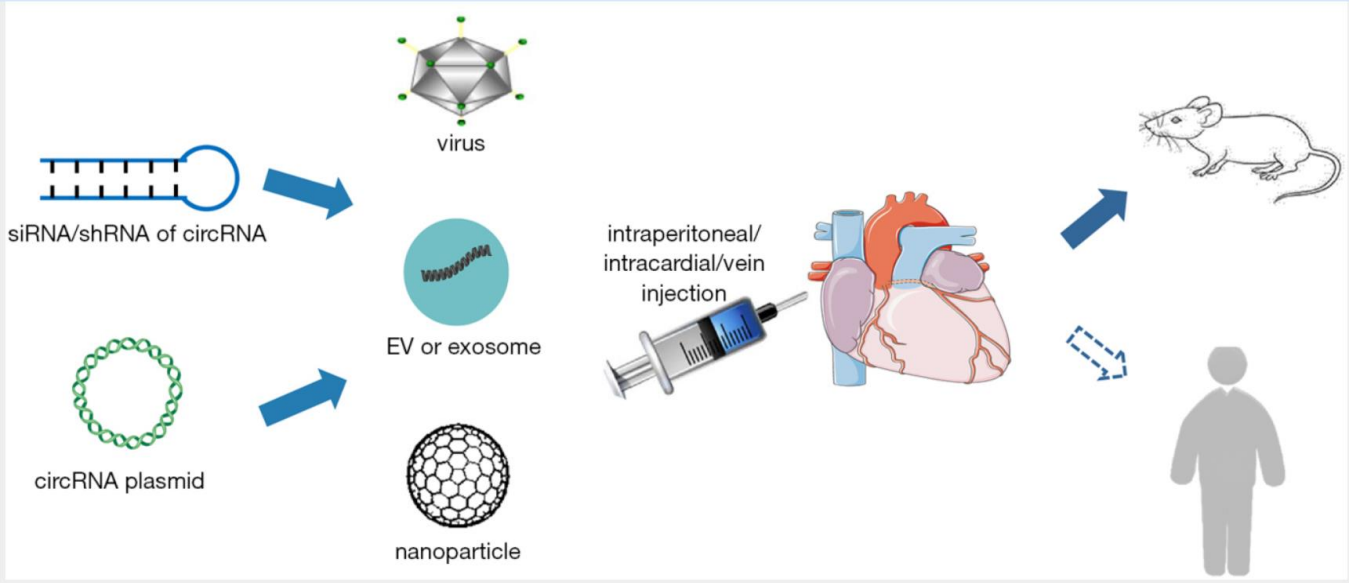

Figure 13: Circular RNA-based treatment approaches for CVDs (Liu et al., 2021)

CircRNA intervention molecules including short-hairpin RNA (shRNA), small interfering RNA (siRNA), or overexpression plasmid, can be delivered *in vivo* via virus, nanoparticle, or other physical or biological materials, exosome or extracellular vesicle (EV) . Intraperitoneal, intracardial, vein injection, and other intervention routes are available.(Liu et al., 2021)

5.1.2. Gene Therapy employing Viral Vectors *In Vivo*

CircRNA treatment based on viral vectors has been used to treat CVDs (Figure 11). Adenoviral vectors have a number of benefits, including transduction productivity as well as high titer, reduced pathogenicity, a broad spectrum of infected tissues, as well as lack of host cell genome

integration. According to a recent study, an isoproterenol-induced mouse model of cardiac hypertrophy and heart failure was treated with an injection of adenoviral circRNA HRCR. This greatly decreased the hypertrophic responses (Wang et al., 2016). Recombinant adeno-associated virus vector (rAAV) has been utilized extensively in gene therapy and vaccination research all over the world due to its benefits of productivity of high transduction, stability, safety, and organ- or tissue -particulated expression. AAV serotypes with distinct tissue affinities include the AAV9 vector, which possesses myocardial tropism and can be utilized to produce a gene unique to the heart (Guenther et al., 2019). AAV9 vectors packaging circRNA were injected intravenously in a study to demonstrate strong circRNA overexpression in mouse hearts (Meganck et al., 2018). *In vivo*, AAV9-related forced overexpression of circSlc8a1 produced HF but RNAi reduction of the very ample circular RNA cricSlc8a1 decreased HF as well as cardiac hypertrophy, according to a study (Lim et al., 2019). According to a study, CircITCH overexpression using an AAV9 vector reduced the symptoms of doxorubicin-induced cardiotoxicity in mice (Han et al., 2020). Additionally, to deliver circFndc3b *in vivo*, it was created AAV9 viral particles indicating circFndc3b downward the CMV promoter modulation. They discovered that when circFndc3b was overexpressed in the hearts of mice after a MI, it decreased cardiomyocyte apoptosis, promoted neovascularization, and further improved heart function (Garikipati et al., 2019). A study also showed that intravenous administration of AAV-9 with sh-circHIPK3 vector two weeks before the implantation of minipumps did, in fact, mute the expression of circHIPK3, and it also reduced the mice's functional impairment as well as Ang II-generated cardiac hypertrophy (Ni et al., 2019). However, because of the induction of genomic integration as well as innate immunological responses, vigilance should still be exercised.

Determining the best virus in the sorroundings of a particular CVD is therefore conducive to minimizing these drawbacks. (Liu et al., 2021)

5.1.3. Exosome- or Extracellular Vesicle-based *In Vivo* Gene Delivery

In a research, circSCMH1-containing extracellular vesicles (EVs) were developed, and it was demonstrated that these EVs significantly sped up the functional restoration of motor function following stroke in mouse and nonhuman primate ischemic stroke models (L. Yang et al., 2020). Target cells can quickly absorb exosomes through particular surface signals. Despite the lack of relevant research, exosomes or EVs altered through cardiovascular cell-particulated surface markers may be a viable circRNA transporter to cure CVDs (Figure 11). However, the strategy is still in its early stages and there are still many unanswered questions, like the ideal dose and administration route. To maximize the therapeutic benefits of this approach, more preclinical or animal studies are required. (Liu et al., 2021)

5.1.4. Nanoparticle-based *In Vivo* Gene Delivery

Nanoparticle-packaged delivery is a promising method to carry out circRNA-based gene therapy because nanoparticles can enhance the transport of drugs to intracellular locations that are difficult to reach by conventional methods (Figure 11). The doxorubicin-induced cardiomyopathy in mice was improved by an intraperitoneal injection of a nanoparticle circAmotl1 plasmid, which was created by packing the circAmotl1 plasmid in polyethylene glycol and combining it with gold nanoparticles (Zeng et al., 2017). However, it is very difficult to generalize this new *in vivo* gene delivery technique because nanotechnology is not particularly well known. Therefore, expanding the use of nanotechnology and interdisciplinary lab collaboration are essential for the future. (Liu et al., 2021)

5.2. **Clinical Significance of CircRNAs in the Diagnosis of Cardiovascular Disease**

The typical diagnostic procedures for cardiovascular diseases are either invasive or non-invasive. For instance, despite being generally accepted, non-invasive procedures like Holter monitoring and electrocardiograms (ECG) have deficient specificityas well as sensitivity. Economically, coronary computed tomography angiography (CTA) is not feasible. An example is that when pathogenic change is just beginning, invasive tests like intravascular ultrasonography as well as coronary arteriography are not helpful. In order to accurately diagnose coronary heart disease (CHD) in the primary stageswhich is non-invasive signature molecule, extremely particular, sensitive, and useful is urgently needed.

If ideal biomarkers are compatible with acurracy, high stability, as well as convenience, they can non-invasive indications of coronary heart disease. Circular RNAs are more stable because of the circular structure, which also increases their half-life in fluids by preventing RNase R-mediated destruction. Additionally, the dynamic and varied expression of circRNAs in a variety of clinical circumstances emphasizes the great sensitivity and specificity of these molecules in disease. Additionally, it has recently been discovered that circRNAs are greatly abundant in the saliva of human, even exosomes as well as bodily fluids. These samples can be collected and extracted using a moderately non-invasive technique. The widespread availability of these samples improves the convenience of detection using a variety of test materials (Bahn et al., 2015; Memczak et al., 2015). Numerous researches have assessed the indicative exhibition of circRNAs in a variety of tumor forms, including hepatocellular carcinoma (ciRS-7) and cholangiocarcinoma (SRY) (Z. J. Zhao & Shen, 2017). Hence it is fascinating to evaluate the

indicative capabilities and also evaluate the clinical utility regarding using circRNAs to identify cardiovascular disease.

The severity and potential fatality of MI as a CAD consequence have long been acknowledged. MI patients are more vulnerable to end-stage HF. For its clinical diagnosis, proteins that are circulating including the brain natriuretic peptide (BNP) are frequently employed. But HF-connected BNP encourages a impeded assessment, that is not optimal regarding early-stage treatment strategy customization (Talwar et al., 2000). A newly discovered circRNA known as MI-related circular RNA (MIRCA) corresponds with post-MI left ventricular dysfunction, according to a blood transcriptome analysis. Patients who are suffer from decreased ejection fraction (EF) of less than forty percent have much lower levels of MIRCA, which aids in a more stratified risk assessment. The rapid dynamic changes in the transcriptome allow MIRCA to happen in a superior diagnostic of differentiating myocardial infraction patients with a prominent chance of progressing HF in comparison with a laggard protein reaction in serum. Additionally, using MIRCA in conjunction with NT-pro-BNP increases the prognostication's trustworthiness in accordance with the Akaike Information Criteria (Salgado-Somoza et al., 2017).

5.3. Functions of CircRNAs in Cardiovascular Disease

5.3.1. Coronary Heart Disease and Atherosclerosis

It has long been recognized that the endothelium plays a crucial role in AS pathophysiology. It is widely known that endothelial damage causes the beginning of AS and promotes the development of atheromatous plaques. Recent reports have identified several circRNAs that are implicated in vascular endothelium dysfunction. One such is the human umbilical vein

endothelial cell (HUVEC) gene hsa circ 0003575, which is expressed more frequently following ox-LDL therapy. HUVEC's apoptosis may have been decreased following hsa circ 0003575 knockdown, according to *in vitro* experiments.

An essential molecular mechanism in AS is VSMC phenotype switching, which fosters cellular proliferation (Bochaton-Piallat & Gabbiani, 2005). The phenotypic change in VSMC through a grownup to proliferating state, which speeds up the onset of AS, is linked with loss of -SMA. Recently, it was shown that circRNAs regulate the expression of their host genes. CircaACTA2 is a prime example (alpha-actin-2). In particular, miR-548f-5p is "sponged" by circACTA2 to increase the mRNA level of its target gene, -SMA (Sun et al., 2017). Additionally, by inhibiting miR-124, circWDR77 promotes VSMC migration and proliferation as well as thickens the vessel wall by increasing the expression of fibroblast growth factor 2(J. Chen et al., 2017).

However, there is insufficient proof of the underlying mechanism (Burd et al., 2010). To better understand the role these diversely transcribed transcripts play in AS, it is intriguing to investigate their functional distinctions. (Gong et al., 2019)

5.3.2. Cardiac Senescence

MFACR, which stands for mitochondrial fission and apoptosis-related circRNA, served as a miRNA sponge for the cardioprotective miR-652-3p, which in turn caused cardiomyocyte cell death and mitochondrial fission. Mitochondrial protein 18 (MTP18) which is another nuclear-encoded mitochondrial membrane protein that promotes mitochondrial fission, is inhibited by microRNA miR-652-3p in cardiomyocytes to prevent mitochondrial fission and apoptosis.

MTP18 is upregulated by CircRNA MFACR, which sequesters miR-652-3p to promote mitochondrial fission including apoptosis (Wang et al., 2017). CircNCX1 is a different circRNA connected to the regulation of apoptosis. Cardiomyocytes express this circRNA in large amounts, and its expression changes in response to apoptotic stress. CircumNCX1 promotes cardiomyocyte cell death by blocking miR-133a-3p's ability to bind to the pro-apoptosis factor cell death-inducing protein (cdip1). The suppression of circNCX1 lowers cardiomyocyte apoptosis after ischemic-reperfusion injury, according to loss-of-function studies (M. Li et al., 2018). In the ischemic myocardium, CircTtc3 is increased, which reduces death of cardiomyocyte cell, according to a recent study. Pro-apoptotic miR-15b is sequestered by CircTtc3, which increases the expression of ADP ribosylation factor-like 2 (Arl2) (Cai et al., 2019) to increase ATP levels and stop cardiomyocyte mitochondria from deteriorating (Nishi et al., 2010).

In a recent study, it was discovered that young and elderly people's peripheral blood showed different levels of circRNA expression (Haque et al., 2020). They then chose 5 circRNAs that were exclusively expressed in each group: young people expressed the following: circITGAX, circPLEKHM1, circDEF6, circATP6V0A1, and circASAP1, aging people expressed the following: circFOXO3, circFNDC3B, circAFF1, circCDYL, and circXPO7, and both groups expressed the following differently: circMIB1, circMETTL3, circEP300 Human senescent cells showed dysregulated expression of the circRNAs FOXO3 and EP300. Meanwhile, hand grip strength, a sign of weakened muscles, is positively correlated with circFNDC3b. Overall, this paper emphasizes the need for additional research into the role of circRNAs and their potential as an aging biomarker (Haque et al., 2020).

5.3.3. Myocardial Infarction

MI is a significant complication of CAD because the coronary artery is suddenly blocked. Cardiomyocytes die as a result of persistent myocardial ischemia, which causes cardiac insufficiency (Takemura et al., 2013). The purpose of conventional treatments is to reperfuse the damaged myocardium in order to protect any remaining cardiomyocytes. Increasing circRNAs have been shown to have an impact on cardiomyocyte apoptosis thus far, making them promising as a brand-new therapeutic target.

Circ-Amot 1 has been discovered using microarray analysis of circRNA profiling. Its host gene, Amotl 1, controls endothelium migration and capillary development, which has an impact on cardiovascular function. Circ-Amot 1 has a preferred expression in neonatal myocardium. According to Yang et al., circ-Amotl1 promotes primary cardiomyocyte survival and lowers apoptosis to provide cardioprotective effects. The inflated left ventricle is rescued, the ejection fraction is increased, and the doxorubicin-induced apoptosis is inhibited by up-regulating circ-Amotl 1 *in vivo*. Circ-Amotl specifically promotes phosphorylated AKT via binding to kinase PDK1 to trigger AKT signaling (Zeng et al., 2017). A renowned circRNA called Cdr1as (or CiRS-7) that was previously examined in the context of neurodevelopment has, on the other hand, been rediscovered as a harmful factor contributing to myocardial dysfunction following MI. As shown by a larger infarct size *in vivo*, increased expression of Cdr1as in cardiomyocytes promotes apoptosis due to activated caspase-3. Such the majority of circRNAs, Cdr1as "sponges" miR-7, which reduces its inhibitory effect on genes involved in hypoxia and apoptosis like SP1 and poly (ADP-ribose) polymerase (Geng et al., 2016).

The energy that mitochondria provide to living cardiomyocytes is crucial for cardiac sufficiency, and cardiac disorders like MI and HF are referred to as a result of their fission and dysfunction (Sabbah et al., 2018). Inhibition of MFACR in mice reduces cardiomyocyte apoptosis and infarct size via alleviating ischemia/reperfusion-induced mitochondrial fission and cardiac dysfunction. The target gene MTP18, a nuclear-encoded mitochondrial membrane protein that promotes mitochondrial fission and cardiomyocyte death, is up-regulated as a result of miR-652-3p being "sponged" by MIRCA (Wang et al., 2017). The regulatory axis of MFACR/miR-652-3p/MTP18 is highlighted in this study as a unique mechanism behind heart injury as well as a potential therapeutic target for cardiac-related disorders.(Gong et al., 2019)

5.3.4. Cardiomyopathy

The term "cardiomyopathy" refers to a variety of myocardial illnesses caused by malfunctions in the contractile machinery, as well as other genes, proteins, and signaling pathways (Fatkin & Graham, 2002; Seidman & Seidman, 2001). A higher risk of developing HF is indicated by cardiac hypertrophy, which involves several signaling cascades. Heart-related circRNA (HRCR) is a newly discovered anti-hypertrophic circRNA that prevents cardiac hypertrophy in a mouse model of isoproterenol-induced hypertrophy as well as heart failure (HF). Specifically, HRCR "sponges" miR-223, which causes its target gene ARC to be upregulated (apoptosis inhibitor with CARD domain). Therefore, in the regulation of HRCR, activated ARC slows the development of cardiac hypertrophy (Gomes et al., 2018; Wang et al., 2016).

Recently, it was shown that many circRNAs were being transcribed from these host genes relevant to cardiomyopathy, and it is hypothesized that the deregulation of circRNA transcription contributes to the development of cardiomyopathy. Computational examination of such

prominent host genes reveals that Titin (Ttn), NPPA, and MYH7 are involved in developmental processes, including controlling cytoskeletal structure, altering myocardial or cellular motility, and packaging myofibrils (Herman et al., 2012; Lamont et al., 2014). The ryanodine receptor 2 (Ryr2), Ppp2r3a, and Slc8a1 are additional host genes implicated with cardiomyopathy (Luo et al., 2010; Y. Yang et al., 2014). Studying their pathogenic potential in cardiomyopathy is advantageous due to their active transcription in illness models (Tan et al., 2017).

Ttn, for instance, acts as a molecular spring throughout the muscle as well as regulates the passive elasticity of the myocardium; its genetic variation could be responsible for dilated cardiomyopathy (DCM) (Okuda et al., 2018; Tayal et al., 2017). Recent research demonstrates that Ttn-generated circRNAs are expressed differently in those patients' left ventricles. A cardiac mutation of the known splicing factor RNA binding sequence protein 20 (RBM20) causes the loss of Ttn-derived circRNAs in DCM models. Given that RBM20 promotes circRNA synthesis by blocking the development of certain pre-mRNA exons as circRNA substrates, RBM20 null animals displayed a down-regulation of Ttn-related circRNAs, an enlarged left ventricle, and impaired cardiac function. Therefore, it seems convincing that preventing the production of certain circRNAs may cause DCM, even though a better comprehension of their overall role is still unclear (Khan et al., 2016). Even though experiments to validate their pathogenic mechanism as well as prospective are lacking, their affluence and close connection with disease-associated host genes warrant additional inquiry (Gong et al., 2019).

5.3.5. Cardiac Fibrosis

Research revealed circRNA 000203 may serve like a miR-26b-5p sponge to encourage the expression of fibrosis-related proteins collagen type I alpha 2 (Col1a2) and connective tissue

growth factor (CTGF), defined as targets of miR-26b-5p, thereby promoting fibrosis. A study discovered that the expression of circRNA 000203 was significantly stimulated in diabetic mouse myocardium and Ang-II-induced mouse cardiac fibroblast (Tang et al., 2017). Correspond to circRNA 000203, circRNA 010567 which found to happen in noticeably stimulated in Ang-II-treated mice cardiac fibroblasts and diabetic mice myocardium. By sponging miR-141, circRNA 010567 promoted the expression of the fibrosis-associated proteins -smooth muscle actin (-SMA), collagen type I as well as collagen type III and transforming growth factor (TGF- β) (B. Zhou & Yu, 2017).

5.3.6. Other Cardiovascular Diseases

Hypertension is a risk factor for cardiovascular disease as well as an aortic aneurysm is a potentially fatal vascular illness characterized by aortic rupture or dissection. Nevertheless, hardly just few circRNA research have been addressed to and thoroughly investigated for these diseases. More study is needed to better comprehend these frequent diseases.

The circRNA microarray revealed that 13 circRNAs were down-regulated in the hypertension group, whereas 46 were up-regulated. Four of them have a considerable expression level, whereas hsa-circ-0005870 has a significant down-regulation level. Despite the lack of additional evidence, gene oncology pathway analysis and the Kyoto Encyclopedia of Genes and Genomes suggest that hsa-circ-0005870 plays a function in hypertension regulation (Wu et al., 2017). In a case analysis of hypertension, researchers discovered that has-circ-0037911 levels were significantly greater in hypertensive individuals than in healthy subjects. There is also a significant connection between has-circ-0037911 and serum creatinine (Scr), supporting the

likelihood of increased expression of has-circ-0037911 incase of hypertensive nephropathy (Bao et al., 2018).

5.4. Circular RNA Stability in Biological Systems

The main characteristic of circRNAs is stability against exonucleases. In situations when the therapeutic drug should be provided less often or in lesser dosages to reduce non-specific adverse effects, highly stable therapeutic RNAs may be useful. Circular RNAs made by endogenous processes are usually two to five times more well-balanced than linear RNAs (Enuka et al., 2016; Jeck & Sharpless, 2014). The significance of these numbers for therapeutic ribonucleic acids delivered ectopically is not yet entirely clear. There are no data describing the stability of circRNAs in living organisms. The stability of synthetic circRNAs in cultured cells *in vitro* has only been studied once so far (Jost et al., 2018): Their half-life ranged from 8 to 20 hours, depending on the delivery method, which can be regarded as brief in comparison to endogenous circRNAs and the half-lives of current linear RNA therapeutics. For instance, after a single injection into the body, antagomiRs, modified antisense DNA- or RNA-like oligonucleotides (ASOs), and double-stranded siRNAs all exhibit *in vivo* consequences that last for ten to fifteen days and as long as several weeks (Bennett & Swayze, 2010; Crooke et al., 2018; Jost et al., 2018). Similar to ASOs, circRNAs may one day be modified to carry various modifications, such as phosphorothioate backbones, 2′,4′-cyclic 2′-O-ethyl modifications, 2′-O-methyl, fluoro, or -O-methoxyethyl conjugates, in order to increase stability, particularly in the extracellular space (Crooke et al., 2018; Krützfeldt et al., 2005). Even so, some modifications may cause local cutaneous reactions or liver and renal toxicity. On the other hand, circRNAs may be chemically altered to theoretically improve other pharmacokinetic characteristics, such as binding to targets and evading immune sensors (Holdt et al., 2018).

Chapter 6 Limitations of CircRNA

CircRNA delivery follows existing methods for therapeutic RNA delivery in theory, as no particular potential or physicochemical barriers have yet been discovered connected with circularity. Delivery methods include local application, subcutaneous injection or depots, and systemic injection into the vasculature. Without a doubt, chemically functionalizing therapeutic RNAs cannot be employed to secure delivery to receptors on intended target organs (e.g., with N-acetylgalactosamine). Local CVD therapy includes covering stents with polymers and hydrogels that encase and discharge therapeutic RNAs at the locations of atherosclerotic lesions. Additionally, current transfection technologies, such photo- or optoacoustic methods that enable target cell identification and cargo delivery through nanostructures, may be modified to disperse circRNAs during cardiovascular procedures. Individual nucleotides may typically enter cells with ease, but circRNAs and circRNA-generating vectors are too large which is greater than1000 Daltons and insufficiently hydrophobic to passively traverse the phospholipid bilayer of the cell membrane. Conversely, endogenously produced circRNAs, like other linear RNAs, are expected to enter target cells via endocytosis. Therefore, prior experience with transfecting nucleic or ribonucleic acids, synthetic, and functionalized nanoparticles, modern lipids, as well as ionizable lipid will be directly beneficial in strategy development for therapeutic circRNA delivery to cells such as through lipid carriers as well as exit from the endosomal membrane since the uptake in the cell. The fact that circular RNAs cannot yet be carried to the nucleus or how the nuclear transit towards the cytoplasm may be managed, despite recent advances in understanding of the nuclear export process for circRNAs (Huang et al., 2018), restricts certain therapy possibilities. (Holdt et al., 2018)

Conventionally, circRNAs can be initiated through direct back-splicing or lariat-driven back-splicing to connect a downstream 3’-terminal and upstream 5’-terminal to generate a circular molecule. CircRNAs are typically stable because of their distinctive circular structure which defends them from exonuclease cleavage. In spite of that, circRNAs are even now degraded, though the degradation mechanisms are unclear. Therefore, studies have suggested various probable approaches that circRNAs can go through degradation:

1) Cleavage through binding of UPF1 and G3BP1

2) Cleavage by RNase P/MRP complex that binds YTHDF2 protein for circRNAs containing m6A modification sites, or

3) Cleavage by AGO2 protein via miRNA-671 sponging. (F. Li et al., 2021)

Identification procedures are yet at their primary period and are influenced by biology-driven methods or microarray analysis. At the current time, they need a careful preparation of the investigational scheme in order to be dependable although next-generation sequencing analysis pipelines demonstrate the potential to enable transcriptome-wide identification of circRNAs. Verification of circRNAs is usually focused on proving the circular nature of the molecule and the existence of the back-splice junction. (Carrara et al., 2018)

Therapeutic strategies based on circRNA have still been investigated throughout preclinical studies thus far. Many hurdles need to be addressed before the therapeutic promise of these techniques can be realized. This section discusses the major drawbacks of these methodologies as well as potential mitigating solutions.

Chapter 7 Future Studies

CircRNAs may one day be used therapeutically in two different ways. One is the therapeutic knockdown or ectopic expression of native disease-linked circRNAs. The creation of synthetic (non-native) circRNAs with desired molecular effects is the second (Figure 12A).

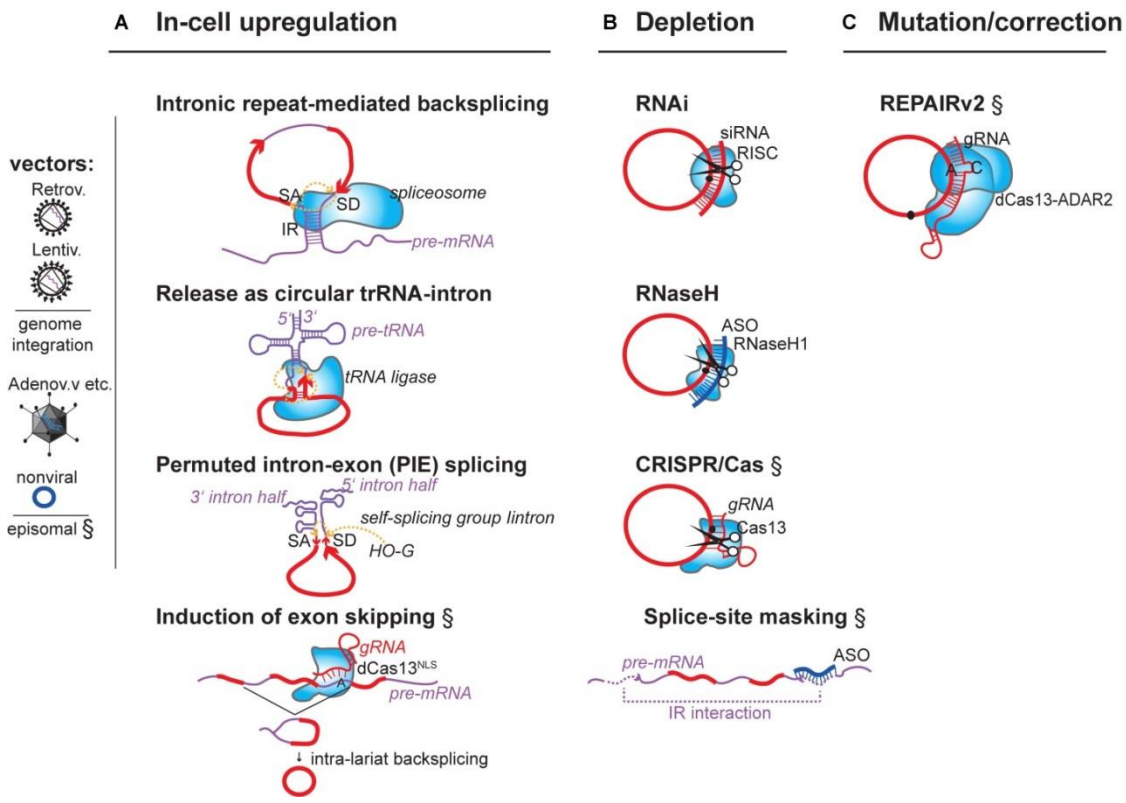

Figure 14: Conceptual overview of mammalian cell-specific endogenous circRNA regulation (Holdt et al., 2018)

The following methods are listed as having not yet been effective in experiments.

A. CircRNA overexpression utilizing minigene designs on RNA-/DNA vectors or by modifying the locus responsible for producing endogenous circRNA.

   i. DNA mini-gene constructs include splice acceptor (SA), splice donor, and transcription start and terminator sites for RNA polymerase II (RNAP II) (SD). The mini-gene constructs have exon(s) that are longer than 300 nucleotides (nts) in length (Barrett et

al., 2015; Kramer et al., 2015) and intronic inverted repeats (IR) (X. O. Zhang et al., 2014a) that are longer than 30–40nts (Liang & Wilusz, 2014) and up to 300–500 nts length (Hansen et al., 2013; Kramer et al., 2015).

ii. The second approach is tRNA intron splicing-mediated RNA circularization. Expression fueled by the U6 promoter and capped by a signal (Lu et al., 2015).

iii. Through intron-group I autocatalytic self-splicing, circularization is the next approach. Even though catalysis still occurs when the catalytic intron is split in half while the sequence of introns and exons is reversed (3′ intron and SA upstream of 5′ intron as well as SD), the mediating exonic order (arrow) in the PIE structure remains circularized in these (permutated) cases, unlike in normal group I splicing. Free guanosine, or G-OH, acts as a hydrophile to start transesterifications (Holdt et al., 2018).

iv. Raising circRNA levels by elevating the frequency of circRNA synthesis. When exon-skipping is elevated, additional circRNAs originating from skipped exons can be produced by post-transcriptional intra-lariat splicing. It is known that skipping is increased when the catalytically inactive nuclear dCas13 variant is targeted by guide RNAs to intronic branchpoint adenosines and/or splice donor sites (Holdt et al., 2018).

B. Depletion methods of circ RNA: Mature circRNAs depletion by

(i) siRNA and shRNA were introduced to the RISC complex. (top)

(ii) By degradation that is reliant on RNase H endonuclease. Through the use of transfected DNA-like antisense oligonucleotides, RNase H may be recruited to circRNAs (ASO, middle), or

(iii) Using CRISPR/Cas nuclease variants (Class 2 CRISPR/Cas13a, b, and d) that target single-stranded RNA. An approach is to mask intronic inverted repeats (IRs) using complementary ASOs in order to inhibit circRNA synthesis (bottom) (Holdt et al., 2018).

C. Correcting/ Mutating circRNA sequence: When a fusion protein made of the catalytically inactive dCas13 nuclease and the ADAR2 adenosine deaminase is expressed, A > I (inosine) can be deaminated at places determined by a guide RNA with a mismatch (C) that links dCas13 to a particular circRNA. Base-pairing between cytidine and inosine perhaps modifying the characteristics of the circRNA at this location (changing binding affinities to other RNAs, DNAs, or proteins). The number of conceivable ribonucleotide conversions will increase when more dCAs13 is fused to various RNA-editing enzymes. Antisense oligos (dark blue), RNA (red), introns (purple), and RNA-binding proteins (light blue shapes).(Holdt et al., 2018)

Chapter 8 Conclusion

Recent investigations have suggested that circRNAs may have a role in the genesis of CVDs. The review thoroughly highlights the research methodology, functional models, and processes of circRNA formation, as well as the numerous circRNAs related to CVDs. Furthermore, it is proposed that developing circRNAs as indicators or circRNA-based therapy methods utilizing biological or physical materials may be advantageous in the future for detecting or treating CVDs.

Remarkably, most analyses only suggested a connection between circRNAs and CVDs, and detailed functions and mechanisms are still in their developing stage, presenting a challenge to developing circRNA-based therapeutic approaches. This is despite the fact that lab work and computational techniques have revealed several circRNAs associated in CVDs. Future circRNA research on CVDs will thus concentrate on the following elements. Initially, *in vitro*, or *in vivo* experiments utilizing extensive bioinformatics and high-throughput sequencing should be used to ascertain the actions of circRNAs. Furthermore, further potential explanations for the function of circRNAs in the genesis and pathogenesis of CVDs must be researched in addition to the miRNA sponge. Last but not least, employing circRNAs as biomarkers or circRNA-based treatment techniques using biological or material components probably lowers the burden of CVD in the population. (Liu et al., 2021)

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