Review Article

Circular noncoding RNAs as potential therapies and circulating biomarkers for cardiovascular diseases

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Abstract
Recent advancements in genome-wide analyses and RNA-sequencing technologies led to the discovery of small noncoding RNAs, such as microRNAs (miRs), as well as both linear long noncoding RNAs (lncRNAs) and circular long noncoding RNAs (circRNAs). The importance of miRs and lncRNAs in the treatment, prognosis and diagnosis of cardiovascular diseases (CVDs) has been extensively reported. We also previously reviewed their implications in therapies and as biomarkers for CVDs. More recently, circRNAs have also emerged as important regulators in CVDs. CircRNAs are circular genome products that are generated by back splicing of specific regions of pre-messenger RNAs (pre-mRNAs). Growing interest in circRNAs led to the discovery of a wide array of their pathophysiological functions. CircRNAs have been shown to be key regulators of CVDs such as myocardial infarction, atherosclerosis, cardiomyopathy and cardiac fibrosis. Accordingly, circRNAs have been recently proposed as potential therapeutic targets and biomarkers for CVDs. In this review, we summarize the current state of the literature on circRNAs, starting with their biogenesis and global mechanisms of actions. We then provide a synopsis of their involvement in various CVDs. Lastly, we emphasize the great potential of circRNAs as biomarkers for the early detection of CVDs, and discuss several patents and recent papers that highlight the utilization of circRNAs as promising biomarkers.

Keywords: atherosclerosis; biomarkers; cardiac fibrosis; cardiomyopathy; circular RNAs; heart failure; myocardial infarction; noncoding RNAs

Introduction
Cardiovascular disease (CVD) is a major killer of the human population in the USA and the world. It is estimated that approximately 100 million American adults (>1 in 3) have ≥1 type of CVD. A total of 11.5% of American adults (27.6 million) have been diagnosed with heart disease. By the year 2030, 43.9% of the US population is predicted to have some form of CVD[1, 2]. With CVD being such a huge burden, advancements in disease management have been directed towards not only the treatment of such diseases, but also the development of platforms for early detection and the possible prevention of CVDs. Over the years, multiple CVD-related biomarkers have been discovered and utilized in clinical settings. According to the American Association for Clinical Chemistry (AACC), common cardiac biomarkers extensively used in the clinic are cardiac troponin and creatine kinase

(CK). Cardiac troponin diagnoses myocardial infarction; its levels are elevated in the blood within 3–4 h upon cardiac injury and remain elevated for 10 to 14 d[3]. CK is mostly used to diagnose a second myocardial infarction occurring shortly after a first one. Blood CK levels are elevated 3–6 h after cardiac injury and remain elevated for 48 to 72 h unless a new injury occurs[4].

With the advancements in technology and the development of deep RNA-sequencing, new components of the genome have been unveiled, including noncoding RNAs. These are regions of the genome that do not code for proteins, but can regulate the function of genes and therefore modulate a vast number of physiological and pathological processes. Based on their function, length and structure, noncoding RNAs are divided into transfer RNAs (tRNAs; 74–95 bp), ribosomal RNAs (rRNAs; 121–5000 bp), small nuclear RNAs (snRNAs; 100–300 bp), small nucleolar RNAs (snoRNAs; 100–300 bp), guide RNAs (gRNAs; 55–70 bp), microRNAs (miRNAs or miRs; 19–23 bp), piwi-interacting RNAs (piRNAs; 24–30 bp), small interfering RNAs (siRNAs; 21–25 bp), long noncoding

RNAs (lncRNAs; >200 bp and linear), and circular noncoding RNAs (circRNAs; >200 bp and circular)[5]. tRNAs were discovered to transfer amino acids to ribosomes during protein synthesis. rRNAs are ribosome components and are directly involved in protein synthesis. snRNAs process mRNA precursors (pre-mRNAs), leading to pre-mRNA splicing and maturation. snoRNAs mediate chemical modifications of other RNAs. gRNAs participate in RNA editing. piRNAs play roles in gametogenesis, transposon silencing, translation suppression, and epigenetic regulation. siRNAs silence complementary target mRNAs. miRs negatively regulate target genes by translational inhibition or promoting mRNA degradation. lncRNAs activate or inhibit gene expression by transcriptional, post-transcriptional, and miR sponge mechanisms, as well as through epigenetic mechanisms to regulate chromatin remodeling. circRNAs regulate alternative splicing and parental gene expression, while also functioning as competing endogenous RNAs or miR sponges[6]. Among the multiple noncoding RNAs, miRs, lncRNAs and circRNAs, in this listed order, have been extensively studied in various pathophysiological processes. Since their discovery, they have become the interest of a large group of researchers and health providers for their tremendous potential as future therapeutics and disease biomarkers.

We have reported some cardioprotective miRs, such as miR-199a-3p, miR-214[7], miR-150[8], miR-125b-5p[9], and miR-532-5p[10]. We have also published multiple reviews on the roles of miRs and lncRNAs, including a review discussing the roles of lncRNAs in CVDs[11] and another review summarizing the literature on the crosstalk between lncRNAs and miRs in health and disease[12].

Viereck *et al* also extensively reviewed the literature and provided the current state of knowledge on circulating noncoding RNAs as biomarkers for cardiovascular disease and injury. Even though the authors mainly focused on miRs and lncRNAs, they briefly discussed circRNAs that could be used as biomarkers for CVDs[13]. This could be attributed to the fact that circRNAs were more recently identified as important reg-

ulators of health and disease; thus, there has not been enough literature showing their involvement in cardiovascular health and disease. The involvement of circRNAs in cardiovascular functions and diseases has been increasingly reported[14]. For example, a recent study by Tan *et al* performed RNA-sequencing on human and mouse hearts as well as on cardiomyocytes derived from embryonic stem cells, and identified over 10 000 circRNAs in the heart[15]. CircRNAs have also been shown to be abundant in tissues and bodily fluids, and are more stable than other noncoding RNAs because their circularization protects them from endonuclease activities[16]. Many review articles including the three described above[11-13] have summarized the importance of miRs and lncRNAs in CVDs. Thus, in this manuscript, we will mainly focus on reviewing studies that identify circRNAs as potential therapies and biomarkers for CVDs; we will also report on the importance of crosstalk between circRNAs and miRs in CVDs.

Circular RNAs as potential therapies and biomarkers for cardiovascular diseases
Circular RNA biogenesis and mechanisms of actions
Biogenesis of circular RNAs

Pre-mRNAs are the precursors of circRNAs, similar to linear mRNAs. Pre-mRNAs are generated by RNA polymerase II (RNA Pol II). If the pre-mRNA is destined to form a linear mRNA, the 2'-OH of a branch point located on an adenine near the 3'-end of an intron performs a nucleophilic attack on the 5'-splice site, forming an intronic lariat and the free 3'-OH of the upstream exon. The free 3'-OH then initiates a nucleophilic attack on the 3'-splice site, joining the exons in the genomic order and releasing the intronic lariat[17].

However, if the pre-mRNA is destined to become a circRNA, the pre-mRNA undergoes a process called "back splicing". Back splicing is different from the "canonical splicing" that forms linear mRNAs (Figure 1). The exact process of back splicing and the formation of circRNAs are not clearly understood. However, a study proposed that the branch point in the upstream intron attacks the 5'-splicing site in the down-
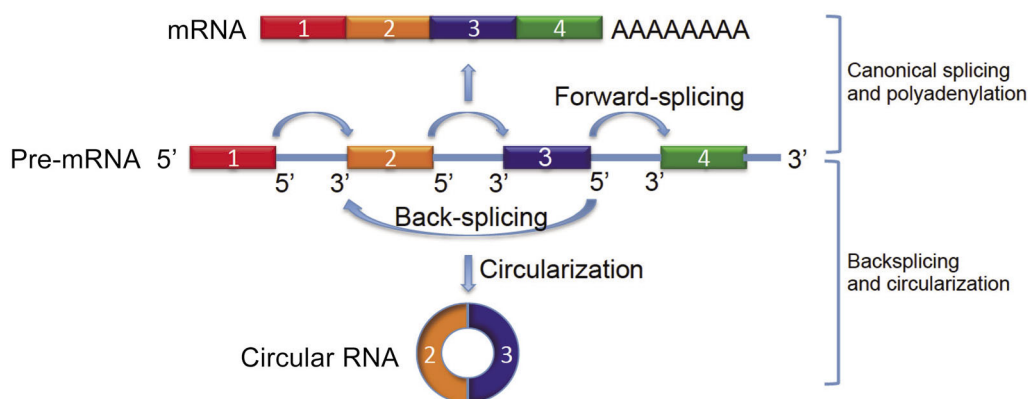

Figure 1. Biogenesis of circular RNAs. Different from linear mRNAs, which are formed by canonical splicing and cutting away introns of the pre-mRNAs using small nuclear ribonucleoproteins (snRNPs), circRNAs are formed by back splicing of the pre-mRNAs and circularization of the cut segment, where the 5' end joins the 3' end.

stream intron, forming a lariat containing the exons[18-20].

Another study suggested the possibility of co-transcriptional back splicing attributed to the following two reasons: (i) nascent chromatin-bound circRNAs[17] and (ii) a change in the ratio of linear mRNAs to circRNAs for individual genes due to changes in the co-transcriptional splicing ratio of RNA Pol II[17]. Although it is not very well understood what could be the trigger for back splicing, two hypotheses have been proposed: (i) Back splicing is associated with Alu elements (approximately 2-fold more) within the intronic regions flanking the exons that form circRNAs. These intronic regions are not only rich in Alu elements, but they are also relatively longer than introns flanking linear mRNA exons[21]. (ii) More recently, a study showed that for back splicing, the 5'-splice site should be rich in the 7-nucleotide GU element, while the branch point should be rich in the 11-nucleotide C element[22].

Depending on their genomic locations, circRNAs are divided into one exonic, multiple exonic, intronic, exon-intronic, intergenic and antisense circRNAs. The expression of circRNAs is tissue- and developmental stage-specific as well as is relatively stable because of their closed ring structure, which prevents them from being degraded by RNA exonucleases[23]. Distinct from classical protein-coding linear mRNAs, circRNAs play regulatory roles by acting as miR sponges[24].

Moreover, circRNAs compete with the linear splicing of pre-mRNAs and regulate the transcription of parental genes[18, 25]. In the following section, we will further discuss the unique roles of circRNAs and how they regulate cardiovascular pathology.

Global mechanisms of circular RNA function
MiRNA sponge:
Cytoplasmic circRNAs are shown to contain binding sites for miRNAs, which are also called miRNA response elements (MREs). This makes circRNAs function as competing endogenous RNAs (ceRNAs)[26]. Because of the ability of circRNAs to bind to miRNAs, they are considered miRNA sponges[23]. Once bound to circRNAs, miRNAs would not be able to bind to their target genes (Figure 2). Because miRNAs exert an inhibitory effect on their target genes, when miRNAs are sponged by circRNAs, their target genes would be upregulated. CircRNAs can possess MREs for multiple miRNAs and can also possess multiple binding sites for an individual miRNA. For example, murine sex-determining region Y (Sry) possesses more than 70 binding sites for miR-7 and has 16 binding sites for miR-138[24].

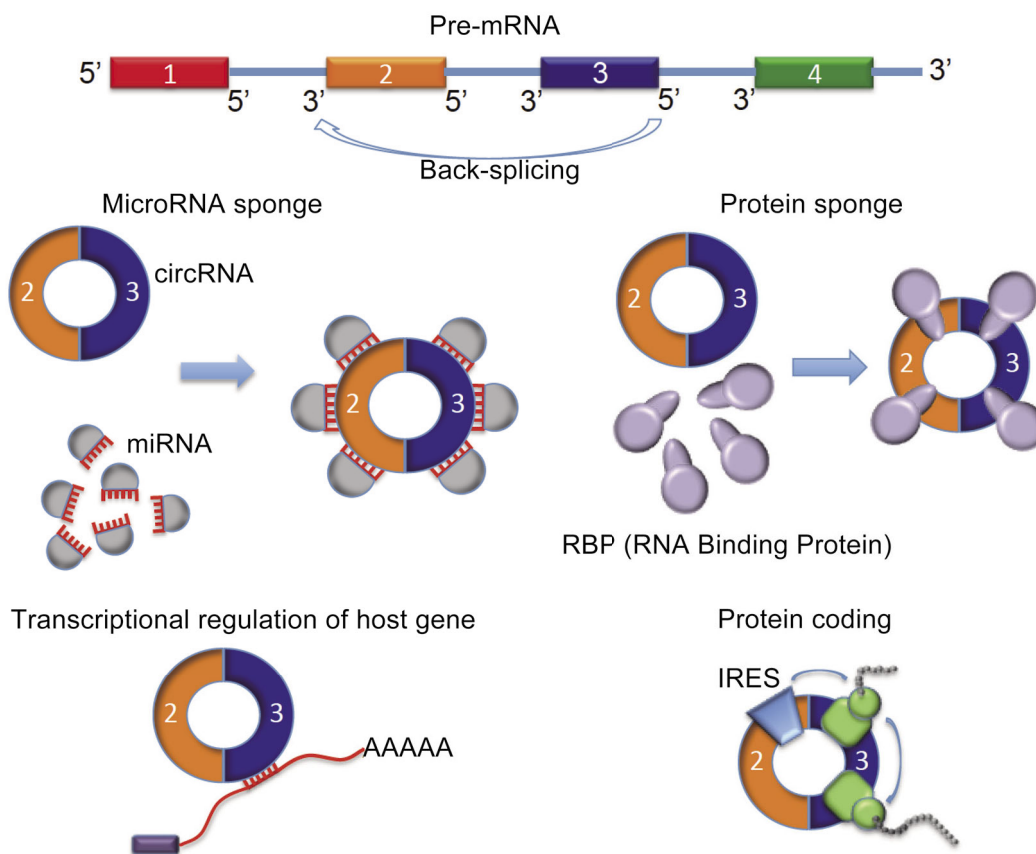Here, we seek to summarize the current knowledge on the interactions of circular RNAs and downstream miRNAs, and to

Figure 2. Mechanisms of actions of circular RNAs. CircRNAs exert their functions through different mechanisms of actions. Some circRNAs bind to miRNAs acting as miRNA sponges, while others bind to proteins acting as protein sponges. Some nuclear circRNAs regulate the transcription of host genes. Lastly, some circRNAs can even code for proteins. IRES: internal ribosome entry site.

Table 1. Circular RNA-microRNA-target gene axis in cardiovascular diseases.

Disease	CircRNA	Target	Regulation	Effect	Reference
Atherosclerosis	CircR-284	MiR-221	↑	Plaque rupture	[32]
Atherosclerosis	CANRIL	Ink4/ARF locus	↑	Increase in risk	[33]
Myocardial infarction	Cdrlas (ciRS-7)	MiR-7a	↑	Apoptosis	[27]
Cardiac fibrosis	Circ_000203	MiR-26b-5p	↑	Cardiac remodeling	[28]
Cardiac fibrosis	CircRNA_010567	MiR-141	↑	Myocardial fibrosis	[29]
Heart failure	HRCR	MiR-223	↓	Cardioprotective	[30]
Cardiac senescence	Circ-Foxo3	ID1/E2F1/FAK/HIF1α	↑	Cardiac stress	[34]

discuss how they work together to regulate the pathogenesis of various CVDs (Table 1). In a paper by Geng *et al*, it was shown that the circRNA CDRlas regulated apoptosis by acting as a sponge for anti-apoptotic miR-7, leading to increased apoptosis[27]. For cardiac fibrosis, circRNAs CircRNA_000203 and CircRNA_010567 have been shown to regulate cardiac fibrosis by sponging miR-26b-5p and miR-141, respectively (Table 1). It is also known that CircRNA_000203 activates cardiac remodeling and that CircRNA_010567 increases the rate of myocardial fibrosis by inhibiting miR functions[28, 29]. CircRNA HRCR can also sponge the deleterious miR-223, leading to cardioprotection against heart failure[30]. A more recent study showed that mitochondrial fission and apoptosis-related circRNA (MFACR) regulates mitochondrial fission and apoptosis in the heart by downregulating miR-652-3p and subsequently upregulating mitochondrial protein 18 kDa (MTP18)[31].

The interactions between circRNAs and miRs are not only important for cardiac diseases but also play a role in vascular diseases (Table 1). For example, it was reported that circRNA CircR-284 regulates the pathogenesis of atherosclerosis by targeting miR-221. By this miR sponging mechanism, CircR-284 increases the risk for plaque rupture[32]. A recent study also showed that circRNAs promote the expression of transient receptor potential cation channel subfamily M member 3 (TRPM3) by inhibiting miR-130a-3p in coronary artery disease patients[35]. Interestingly, a systematic study on circRNA expression profiles in human adult and fetal tissues suggests that circRNAs exhibit their roles in a tissue- and development-specific manner and that investigation of the circRNA-miR-mRNA network will provide important insights into therapeutic and diagnostic targets[36].

Protein sponge:
CircRNAs not only possess miRNA-binding elements but also possess binding sites for proteins. When circRNAs bind to their target proteins, this will lead to the inhibition of protein function (Figure 2). An example is muscle blind (MBL) protein, which is a critical factor for the alternative splicing of mbl pre-mRNA. CircMbl possesses binding sites for MBL protein and can bind to that protein, leading to inhibition of mbl pre-mRNA alternative splicing. It is worth noting that there is a feedback loop mechanism in this process as proposed by Ashwal-Fluss *et al*. This feedback loop entails that when MBL protein is abundant, mbl pre-mRNA is spliced into circMbl, which binds to MBL and decreases its bioavailability[18].

Transcriptional host gene regulation:
CircRNAs are released into the cytoplasm and even extracellularly to have the potential to serve as biomarkers. However, to a much lesser extent, a few circRNAs remain in the nucleus. These nuclear circRNAs can react with RNA Pol II in the promoter region of host genes (Figure 2) and regulate transcription[22, 37]. Examples of these circRNAs are exon-intron circRNAs (EIciRNAs) and circular intronic RNAs (ciRNAs). EIciRNAs such as CircEIF3J and CircPAIP2 possess binding sites for the U1 small nuclear ribonucleoprotein (snRNP) in the nucleus and have the ability to bind to U1 snRNP, forming the EIciRNA-U1 snRNP complex. This complex can then interact with RNA Pol II in the gene promoter region, which enhances the transcription of their parental genes[25, 37].

Protein coding:
For a gene to be translated into a protein, the formation of a translation initiation complex is critical. The assembly of this complex requires the recognition of the 5'-cap structure at the 5'-end of mRNAs[38]. CircRNAs do not possess a 5'-cap, and therefore do not normally code for proteins. However, CircZNF609 in human muscle cells[39] and CircMbl in fruit fly head[40] have been recently reported to encode proteins. Both of these circRNAs were able to interact with ribosomes and code for proteins in a 5'-cap-independent manner (Figure 2). The mechanism for 5'-cap-independent protein coding was explained by Yang *et al* as occurring as a result of N6-methyl-adenosine (m6A) modifications near the circRNA start codon. This m6A modification can act as an internal ribosome entry site (IRES) and lead to translation initiation[41].

Identification of circular RNAs as important regulators of cardiovascular development and disease

CircRNAs were initially discovered in the early 1990s[42] and were thought to be generated by RNA splicing errors or be splicing process by-products at that time. In 2013, circRNAs were revealed to function as molecular sponges for miRs[24], making circRNAs a new hotspot in the noncoding RNA field after miRs and lncRNAs.

Due to advanced sequencing technologies and data analysis

methods, a growing number of circRNAs have been identified in human and murine hearts[15, 43, 44]. The first circRNA profiling study discovered 575 circRNAs from adult murine hearts and demonstrated that several circRNAs are involved in CVDs[43]. The systemic analysis of circRNA expression from rats (neonatal *vs* adult), mice (sham *vs* transverse aortic constriction) and humans (non-failing *vs* failing) identified over 9,000 circRNAs from each species[44]. More recently, a detailed annotation and analysis of genome-wide circRNA expression reported 15 318 circRNAs in human hearts[15]. Even though the aforementioned studies are descriptive and somehow coarse in their designs, they have provided a very important basis for the discovery of novel circRNAs that could be translated clinically as either disease biomarkers or therapeutic agents for CVDs.

There has been significant effort in establishing bioinformatics tools to obtain insights into the roles of the identified cardiac circRNAs. For example, Liu *et al* reported a circRNA database (http://circnet.mbc.nctu.edu.tw/)[45], in which researchers could access information about tissue-specific circRNA expression profiles and target information for circRNAs of interest[45, 46]. Several other circRNA databases have also been constructed, including circBase (http://circbase.org/)[47], CircRNABase (http://starbase.sysu.edu.cn/mirCircRNA.php)[48], Circ2Traits (http://gyanxet-β.com/circdb/)[49], and CircInteractome (http://circinteractome.nia.nih.gov)[50]. These databases provide excellent platforms to facilitate further functional research on circRNAs. However, the predictive bioinformatics data herein should be experimentally validated before pursuing any functional studies. For example, a novel circRNA should be subjected to extensive validation of its circularity using RNase R digestion, assessment by PCR using divergent primers and Sanger sequencing of the junction region. Quantitative PCR analysis remains the most widely used technique to assess the levels of circRNAs. In the next section, we will discuss how circRNAs are identified and assessed in various CVDs by distinct diseases.

Circular RNAs in cardiac development
The importance of noncoding RNAs in the gene regulatory network of cardiac development has been studied and demonstrated in multiple studies[51-53]. It has been recently shown that not only does alternative splicing regulate cardiac development, but back splicing can also play a critical role in cardiac development[54]. A study by Werfel *et al* also identified that circRNAs are highly expressed during cardiac development using cardiac tissues from humans and rats as well as human embryonic stem cells that were differentiated to cardiomyocytes. Even though this study was descriptive in nature and lacked mechanistic details, it was able to identify some circRNAs that potentially play important roles in cardiac development. According to the study, the most abundantly expressed circRNAs in the heart tissues were circRNA slc8a1 (Table 2) and circRNA rhobtb3[44]. Another study by Tan *et al* showed similar results[15]. Interestingly, *Slc8a1* is known to be a sodium/calcium exchanger that is critical for heart development and contraction[55]. *Rhobtb3* is a Rab9-regulated ATPase required for endosome to Golgi transport and is highly expressed in the heart[56]. A study focused on a human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) model of cardiomyopathy also uncovered a new signature of disease-relevant circRNAs, including circRNAs from genes that are known regulators in cardiac development and function (*eg*, *atxn10, chd7, dnajc6* and *slc8a1*)[57]. Lastly, a recent functional enrichment study showed that circRNAs play important roles in pathways that are specifically activated during cardiac differentiation[58].

Circular RNAs in vascular development
CircRNAs' roles in embryonic development extend to vascular development. A study by Boeckel *et al* demonstrated that circRNA cZNF292 has a critical role in promoting angiogenesis (Table 2). CircRNA cZNF292 is expressed in endothelial cells in the human umbilical vein, microvessels and aorta. When endothelial cells were exposed to hypoxia, the expression of circRNA cZNF292 was increased, while *in vitro* silencing of cZNF292 inhibited the normal functional characteristics of endothelial cells, such as tube formation and spheroid sprouting, suggesting pro-angiogenic roles for circRNA cZNF292[59].

Roles of circular RNAs in myocardial infarction
Myocardial infarction (MI) is defined as damage and remodel-

Table 2. Circular RNAs associated with cardiovascular development and disease.

CircRNA	Cardiovascular involvement	Regulation	Reference
CircSLC8a1	Heart development	Unknown	[44]
CDR1as	Myocardial infarction	↑	[27]
MICRA	Myocardial infarction	Unknown	[60]
HRCR	Hypertrophic cardiomyopathy	↓	[30]
CircTitin	Dilated cardiomyopathy	Unknown	[61]
CircRNA_000203	Cardiac fibrosis	↑	[28]
CircFoxo3	Myocardial senescence	↑	[34]
CZNF292	Angiogenesis	↑	[59]
CircANRIL	Atherosclerosis	↓	[62]
Circ_0124644	Coronary artery disease	Unknown	[63]

ing of cardiac tissue in response to decreased blood flow in coronary arteries. It is usually predisposed by atherosclerosis and leads to the occlusion of coronaries. MI eventually leads to cardiac dysfunction, heart failure and ultimately death. MI treatment is directed towards reducing post-infarction cardiac remodeling, which prevents heart failure[64]. Since the discovery of noncoding RNAs, they have been regarded as potential therapeutics for MI[65]. Using microarray analysis, Wu *et al*[66] discovered 63 circRNA candidates, including circRNA CDR1as, that are differentially expressed in normal murine myocardial tissues compared to failing myocardial tissues due to MI. Simultaneously, another study by Geng *et al*[27] reported the circRNA CDR1as as a mechanism of action in MI injury (Table 2) and showed that MI induces the upregulation of CDR1as, which in turn promotes cardiomyocyte apoptosis by acting as a sponge for cardioprotective miR-7a (Table 1).

Roles of circular RNAs in ischemia-reperfusion injury

Ischemia-reperfusion injury is damage occurring to vital organs, such as heart, kidney and brain, due to the transient decrease in blood flow to these organs[67]. In a study by Mehta *et al*[68], the authors subjected mice to transient middle cerebral artery occlusion and performed circRNA expression profiling in the penumbral cortex at 6, 12, and 24 h of reperfusion using circRNA microarray analysis. They first found over 1000 circRNAs to be expressed at detectable levels from mostly exonic regions of genes in the cerebral cortex of sham animals. Among them, 283 circRNAs were expressed higher than 2-fold during at least at one of the reperfusion time points, whereas 16 were altered at all 3 reperfusion time points after the transient middle cerebral artery occlusion. The authors also used bioinformatics analysis to show that these 16 circRNAs contain binding sites for many miRs, although they did not experimentally validate the target miRs.

Roles of circular RNAs in cardiomyopathy

Cardiomyopathy, including hypertrophic and dilated cardiomyopathy, is a cardiac pathology that affects the myocardium[69]. Hypertrophic cardiomyopathy (HCM) occurs when the left ventricular myocardium becomes thicker than normal, leading to incomplete filling at diastole. This eventually leads to heart failure and death. Dilated cardiomyopathy (DCM) occurs when the left or right ventricles are impaired, leading to ventricular dilation, heart failure and death. Wang *et al* reported that the heart-related circRNA HRCR confers protective effects against myocardial hypertrophy and heart failure[30]. In this study, when mice were infused with isoproterenol and subjected to transverse aortic constriction, HRCR expression was downregulated (Table 2). Using bioinformatics prediction, co-immunoprecipitation and AGO2 immunoprecipitation analyses, the study demonstrated that HRCR interacts with miR-223, retains this miR in the cytoplasm, and inhibits the pro-hypertrophic activity of miR-223 (Table 1). Another study by Khan *et al*[61] reported abnormal circRNA splicing events during cardiomyopathy. Using RNA-sequencing analysis on heart samples from HCM and DCM patients,

the authors identified over 800 back splicing junctions. They validated the profiling results using QRT-PCR analyses and confirmed that 10 exonic *calcium/calmodulin-dependent protein kinase type II delta* (*camk2d*) and *titin* circRNAs were associated with cardiomyopathy. *Camk2d* circRNAs were downregulated in both HCM and DCM patients, while *titin* circRNAs were downregulated only in DCM. A profiling and validation study also provided a comprehensive catalogue of RNase R-resistant 575 circRNAs in adult murine hearts that may play important roles in cardiomyopathy[43].

Roles of circular RNAs in cardiac fibrosis

Cardiac fibrosis is a cardiac pathology characterized by the activation of cardiac fibroblasts, leading to the replacement of healthy myocardium with non-beating fibrous tissues[70]. Cardiac fibrosis occurs as a part in the pathogenesis of various CVDs, such as MI and diabetes mellitus. A study by Tang *et al* showed that CircRNA_000203 back spliced from the mouse *myo9a* gene is associated with cardiac fibrosis[28]. Diabetic mouse myocardium and angiotensin II-induced cardiac fibroblasts showed CircRNA_000203 upregulation (Table 2). The study also identified anti-fibrotic miR-26b-5p to be a direct target for circRNA_000203 (Table 1). If miR-26b-5p is sponged by CircRNA_000203, cardiac fibroblast proliferation is enhanced via the activation of collagen type I a2 (Col1a2) and connective tissue growth factor. CircActa2 has also been reported to regulate the expression and function of alpha smooth muscle actin (α-SMA; the main player in myocardial and endocardial fibrosis) via sponging miR-548f-5p[71, 72].

Roles of circular RNAs in cardiac senescence

The turnover of adult cardiomyocytes is very low and is not sufficient to overcome cardiac damage once they are differentiated into their mature forms. Cardiomyocytes also undergo pathological aging termed cardiac senescence. Du *et al* reported that exonic CircFoxo3 could induce cardiac senescence[34]. Exogenous CircFoxo3 could aggravate heart senescence and doxorubicin-induced cardiomyopathy. This could be reversed by knockdown of CircFoxo3 (Table 2). The study also provided mechanistic insights showing that CircFoxo3 promotes cardiac senescence by binding to inhibitor of differentiation 1 (ID1), transcription factor E2F1, focal adhesion kinase (FAK) and hypoxia-inducible factor 1α (HIF1α). Once bound to CircFoxo3, the aforementioned factors remain in the cytoplasm, leading to cardiac stress (Table 1).

Roles of circular RNAs in atherosclerosis

Atherosclerosis is a vascular disease caused by injury to the vascular endothelium that leads to the accumulation of cholesterol that eventually blocks the vessel. MI usually occurs when a blood clot blocks blood flow to the heart. Atherosclerosis could have a genetic predisposition due to its association with the *ink4/arf* locus on human chromosome 9p21, where several tumor suppressor genes (*p16ink4a*, *p15ink4b* and *arf*) and the lncRNA ANRIL are transcribed. Several circular transcripts of *anril* have been reported, and their expression is shown to

correlate with atherosclerosis risk[14, 33]. Holdt *et al* showed the molecular mechanism of one of the circular ANRIL isoforms[62]. CircANRIL impaired the maturation of ribosomes by binding to the 60S ribosomal assembly factor PES1, leading to nuclear stress and p53 activation (Table 2). This eventually results in the inactivation of smooth muscle cells and macrophages while inducing apoptosis. Thus, CircANRIL could be protective against atherosclerosis.

Circular RNAs as novel biomarkers for cardiovascular diseases

Of all noncoding RNAs, circRNAs could be the best candidates as future disease biomarkers for the following reasons. First, the circular 3'- and 5'-ends of circRNAs are not exposed to degradation by exoribonucleases (RNases), and thus their half-lives are longer upon extracellular release. This makes circRNAs more stable and abundant in extracellular fluids for easy detection. Second, deep RNA-sequencing studies have identified hundreds to thousands of cell- and/or tissue-specific circRNAs in human and mouse[73]. Third, circRNAs are present in the whole blood, plasma and extracellular vesicles such as exosomes[60, 74, 75], indicating that most components of the blood (*eg*, peripheral blood mononuclear cells, exosomes, platelets, plasma and serum) can be used for biomarker studies.

For example, an interesting study on human platelets showed that circRNAs are enriched in platelets 14- to 26-fold relative to samples digested with RNase R to selectively remove linear RNAs, suggesting that linear RNAs decay more rapidly than circRNAs in platelets[76]. CircRNAs are also abundant in exosomes or microvesicles, which are secreted into the extracellular fluid by cells. The abundance of circRNAs in exosomes is positively correlated with their cellular expression[77]. Many studies have already reported circRNAs as biomarkers in some diseases, mainly cancer. For example, a study by Chen *et al* showed Circ_0000190 to be downregulated in human gastric cancers[78], while Xu *et al* identified CircRNA ciRs-7 (CDR1as) as a risk factor for microvascular invasion in hepatocellular carcinoma[79]. CircRNAs not only serve as disease biomarkers for cancers but can also act as biomarkers for other diseases such as neurological disorders. For example, a study by Floris *et al* showed CircRNA_103636 to be downregulated in major human neurological disorders[80]. Another study by Iparraguirre *et al* showed that Circ_0005402 and Circ_0035560 are back spliced from *anxa2* and downregulated in multiple sclerosis[81]. More recent work by Maass *et al* also highlighted the promise of circRNAs as tissue- and disease-specific markers[82].

Like the aforementioned diseases, CVDs are associated with the dysregulated expression of circRNAs. Accordingly, there has been increasing interest in circRNAs as potential biomarkers for CVDs. A study by Zhao *et al* reported Circ_0124644 to be upregulated in the bloodstream in patients with coronary artery disease and proposed Circ_0124644 as a potential clinical biomarker for coronary artery disease[63] (Table 2). Another study showed that a circular RNA called myocardial infarction-associated circular RNA (MICRA) back spliced

from exon 1 of zinc finger protein 609 as a predictor of left ventricular dysfunction after acute MI[60] (Table 2). In this case, the blood levels of MICRA in MI patients were lower than in healthy people. MI patients with relatively low levels of MICRA are considered at high risk of left ventricular dysfunction. A study by Bazan *et al* also described the role of CircRNA-284 as a biomarker for carotid plaque rupture[32] (Table 1). CircRNA-284 is an inhibitor of miR-221 and miR-222, which are indicators of carotid plaque rupture. Thus, increased blood levels of CircRNA-284, and decreased blood levels of miR-221 and miR-222 could be utilized as a biomarker for carotid plaque rupture. Although there have been not many reports on circRNAs as potential biomarkers for CVDs, the utility of circRNAs as CVD biomarkers will significantly increase due to their superior stability compared with other noncoding RNAs.

Review of recent patents

Even though the identification of circRNAs as biomarkers for CVDs is pretty recent, one can already find multiple patents filed on multiple circRNAs as potential CVD biomarkers. In this section, we review some of these patents that present methods and techniques for the detection of aberrantly expressed circRNAs as well as their utilities for the diagnosis and prognosis of heart diseases. These circRNAs could be used for new types of biomarkers that lead to novel diagnostic, prognostic and predictive platforms.

Patents EP3054017 A1 and WO2016124655 A1 reported 16 circRNA transcripts that are correlated with CVDs, in particular, acute myocardial infarction (Table 3). It is proposed that the circRNA transcripts in these patents could serve as biomarkers for the diagnosis of cardiovascular disorders. The patents also provide diagnostic kits for the dysregulated circRNAs in patients with CVDs. The patents also discuss the effects of these circRNAs on endothelial cell sprouting and pathological angiogenesis. Lastly, the patents discuss that compounds or vascular tissue materials, which have been used to treat CVDs and endothelial dysfunction, could regulate the identified 16 circRNAs[83, 84]. Patent WO2017046203 A1 (Table 3) also demonstrated Circ_0000615 (MICRA), Circ_0000005, Circ_0000673, Circ_0000585, Circ_0000816, Circ_0000817, Circ_0000917, Circ_0001423, Circ_0000540, Circ_0001844, Circ_0000994 and CircNPPA to be dysregulated in heart failure and myocardial hypertrophy, and thus can be used as biomarkers for these diseases. This patent claims the utility of the identified circRNAs in the diagnosis of heart failure and describes kits designed to diagnose MI using quantitative expression data for one or more of the identified circRNAs. Lastly, the patent discusses the current pharmacotherapy that regulates the identified circRNAs[85]. Finally, patent WO2012050975 A2 discovered circRNAs generated from one or more *anril* exons (Table 3). The patent also provided methods for detecting Circ-ANRIL in multiple disorders, such as atherosclerosis, and information about related kits. Lastly, the patent discusses methods for screening for compounds to prevent or treat such disorders[86].

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Table 3. Patented circular RNAs as potential biomarkers in cardiovascular diseases.

Disease	CircRNA	Patent	Reference
Myocardial Infarction	U1-U7 and D1-D9	EP3054017 A1 WO2016124655 A1	[83, 84]
Heart failure	MICRA, circ_0000005, circ_0000673, circ_0000585, circ_0000816, circ_0000817, circ_0000917, circ_0001423, circ_0000540, circ_0001844, circ_0000994, and circNPPA	WO2017046203 A1	[85]
Atherosclerosis	circ-ANRIL	WO2012050975 A2	[86]

Conclusions and current & future developments

Very little is known about circRNAs and how they function and regulate multiple physiological and pathological processes. This certainly would delay the utilization of circRNAs in the clinical setting until enough knowledge is established about them[16, 87]. All of the previous studies involving human subjects contain only a small cohort of patients or healthy subjects, making the studies inconclusive. Accordingly, the field is in need for studies with much larger cohorts that can provide more convincing data on pathophysiological roles of circRNAs and their potential utilities as biomarkers. Moreover, there is often a large discrepancy in the results between different groups and a lack of data reproducibility, which is mainly attributed to the absence of standardized methodologies for measuring the levels of circRNAs in body fluids and tissues. The aforementioned limitations make the translation of circRNAs as biomarkers for CVDs to the clinic somehow risky and not currently favorable for FDA approval.

However, circular RNAs are increasingly being recognized to play important roles in the evolving strategies for tackling CVDs as described above. The recent studies and patents in the circular RNA field described in this review also illustrate the tremendous promise of circRNAs in biomarker research. Although some circular RNA biomarkers for heart disease have been identified, there remain significant hurdles with respect to clinical use due to diagnostic limitations and off-target effects. More work is needed to better validate and integrate circRNAs into clinical practice. For example, many research directions may help deepen our knowledge of the roles of circRNAs in CVDs and addressing their value as biomarkers. (i) A detailed characterization of circRNA biogenesis is warranted. (ii) Profiling analyses focused on the temporal-, cell- and tissue-specific expression of circRNAs in healthy and diseased tissues would provide important insights, as the originating tissues for circulating circRNAs are unknown and it is possible that they can be taken up by other tissues, leading to secondary effects. (iii) A characterization of the full spectrum of circulating circRNAs in the bloodstream would guide in selecting candidate circRNAs for future biomarker studies. (iv) The identification of the best components of blood for circRNA detection would be needed for developing clinically applicable diagnostics for circRNAs. In conclusion, collaborative approaches between basic scientists and clinicians will be required to develop circRNA diagnostic or therapeutic modalities.

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