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Review Article

Expanding roles of circRNAs in cardiovascular diseases

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ABSTRACT

CircRNAs are a class of single-stranded RNAs characterized by covalently looped structures. Emerging advances have promoted our understanding of circRNA biogenesis, nuclear export, biological functions, and functional mechanisms. Roles of circRNAs in diverse diseases have been increasingly recognized in the past decade, with novel approaches in bioinformatics analysis and new strategies in modulating circRNA levels, which have made circRNAs the hot spot for therapeutic applications. Moreover, due to the intrinsic features of circRNAs such as high stability, conservation, and tissue-/stage-specific expression, circRNAs are believed to be promising prognostic and diagnostic markers for diseases. Aiming cardiovascular disease (CVD), one of the leading causes of mortality worldwide, we briefly summarize the current understanding of circRNAs, provide the recent progress in circRNA functions and functional mechanisms in CVD, and discuss the future perspectives both in circRNA research and therapeutics based on existing knowledge.

1. Introduction

Circular RNAs (circRNAs) are ubiquitous, highly stable, and evolutionarily conserved RNA molecules among eukaryotes [1]. Structurally, circRNAs are more resistant to exonucleases than linear RNAs because the lack of 5′ to 3’ ends with covalently looped structures [2]. This unique structure suggests that circRNA may more stably exert its functions inside the cells and deliver biological messages in cell-cell communications [3]. The expression of circRNAs is tissue- and cell type-specific and is specifically regulated during normal physiology and disease progression [4]. In the past decade, circRNAs have continuously been found to function in multiple physiopathological processes through multiple mechanisms [5–8].

For decades, circRNAs were considered to be by-products of RNA splicing or so-called “scrambled exons” in humans [9,10]. However, advanced next-generation sequencing techniques in the early 2010s and circRNA-specific bioinformatics algorithms have been developed to preserve non-polyadenylated RNAs, which revealed that thousands of ubiquitously expressed and covalently closed noncoding RNAs were systematically detected in many species, including human, mouse and rat [5,11–13]. Further studies confirm that circRNAs exhibit disease- and tissue-specific expression patterns, multiple functions to participate in disease pathogenesis, and extraordinary potential for therapeutic applications [14,15].

In this review, we present an overview of recent advances in circRNA biology and strategies to study circRNAs, and highlight the current knowledge of their functional roles in several major cardiovascular diseases. We also outline the perspectives for the therapeutic and diagnostic potential of circRNAs.

2. Overview of circRNAs

2.1. Classes of circRNAs and the biogenesis

CircRNAs are derived from linear precursor mRNAs (pre-mRNAs) transcribed by RNA polymerase II, which endows them with a unique closed continuous ring structure site [2,16,17]. CircRNAs can be generated via multiple mechanisms (Fig. 1). For example, flanking intron pairing [18], RNA-binding protein (RBP) [19], and lariat across introns [20] can facilitate circRNA biogenesis by bringing the distal flank of back-spliced exons into proximity. Based on different subcellular localization and sequence composition, circRNAs can be divided

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into four categories: exonic circRNAs (EcircRNAs) that are predominantly localized in the cytoplasm [21], intronic circRNAs (ciRNAs) [22] and exon-intron circRNAs (EIcircRNAs) in the nucleus [23], mitochondria-encoded circRNAs (mecciRNAs) that are encoded by mitochondrion genome generated via a splicing-independent mechanism [23,24]. Several factors may influence the alternative splicing and biogenesis of circRNAs [6]. Abundant circRNAs tend to be derived from genes with highly active promoters [25,26]. Epigenetic modifications within histones and gene bodies may also affect circRNA biogenesis [27], and circRNAs may directly affect the epigenetic status of host gene promoter regions [28].

2.2. CircRNA nuclear transport and subcellular localization

Although some circRNAs are retained in the nucleus (e.g. EIciRNAs and ciRNAs), most circRNAs are localized in the cytoplasm [29]. They are generated in the nucleus and are exported to the cytoplasm for the function via certain mechanisms. The nuclear export of mature RNA transcripts is critical for their proper biological functions. In the past decade, limited evidence indicates how circRNAs are exported from the nucleus such as length-dependent nuclear export [30], m6A-mediated nuclear export [31,32], and intercellular transport of circRNAs via extracellular exosomes [33]. For example, relatively long circRNAs (>800-nucleotide) require DExH/D box RNA helicase Hel25E for nuclear export in *Drosophila* cells [30]. Recently, we have provided lines of evidence for the evolutionarily conserved Exportin 4 (XPO4) in the nuclear export of EcircRNAs. Depletion of XPO4 leads to nuclear accumulation of EcircRNAs which generates R-loop formation and DNA damage, leading to fertility defects and neurological disorders [34]. The nuclear export patterns of these circRNAs are still under investigation, and future studies should provide critical insight into whether unbalanced circRNA subcellular localization and inappropriate nuclear export play a role in cellular fitness and homeostasis, as described in cancer [35,36].

Several approaches have been applied in the determination of circRNA subcellular localization. Initially, circRNA localization can be determined by nucleocytoplasmic fractionation followed by RT-qPCR and droplet digital PCR, which can also be used for absolute quantification of circRNAs and scored as positive or negative based on the fluorescence signal [37]. For high-throughput circRNA quantification, NanoString Technologies' nCounter platform can be used, which is based on digital single-molecule counting using an automated fluorescence microscope [38]. RNA fluorescence *in situ* hybridization (FISH) can quantify circRNAs and display the cellular localization of circRNAs using DNA probes targeting circRNA junction sites [39,40]. Ribosome profiling (RIBO-seq) uses deep sequencing of ribosome-protected mRNA fragments to monitor translation with speed, accuracy, and scale unmatched by mRNA-level monitoring methods [41]. As RIBO-seq advances, more circRNAs are being found to be translated in different organisms, and a subset of them can produce functional polypeptides [42]. Additionally, cytoplasmic and nuclear circRNAs can be biochemically isolated in different proportions and subsequently analyzed for different circRNA species on a genome-wide level using high-throughput approaches. For example, CeFra-seq was used to localize RNAs across multiple cell fractions [43], while APEX-seq examined extensive patterns of localizing different categories of circRNAs at multiple subcellular locations [44]. In addition, several databases have been established to provide a genome-wide summary of the subcellular localization of circRNAs, including CircVIS [45], MNDR v3.0 [46], and RNALocate [47]. Several artificial intelligence (AI) techniques were also developed to predict circRNA subcellular localization, including Circ-LocNet [48], mRNALoc [49], and RNAlight [50].

2.3. Biological functions for circRNAs

CircRNAs have been suggested to have multiple functions. Nuclear circRNAs modulate gene expression by regulating transcription or binding to protein factors [40], while cytoplasmic circRNAs function in various organisms such as acting as microRNA sponges or competing endogenous RNA (ceRNA) [51], and even translation templates [21]. Furthermore, the exceptional tertiary structure of circRNAs provides them with significant flexibility for RNA-binding protein (RBP) binding [52]. Additionally, circRNAs can also bind to mRNAs for the regulation of mRNA stability and translation [53]. This mode of action enables the regulation of mRNA translational adaptability, which in turn affects gene expression in various physiological processes [54]. Some circRNAs have been identified as transcripts capable of cap-independent translation [55]. The need for further research and application of circRNA in disease treatment is emphasized by the growing understanding of the complex functions of circRNA.

3. Role of circRNAs in cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of mortality globally [56,57]. CVDs are commonly referred to as a class of diseases in the heart or blood vessels [58], including, coronary artery diseases (e.g.

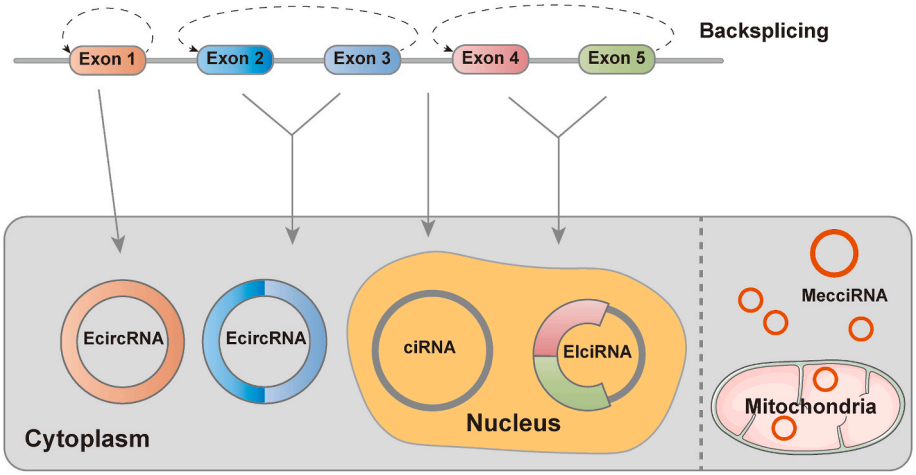

Fig. 1. CircRNA biogenesis and subclasses. Nuclear genome-derived circRNAs comprise three groups: Exonic circRNAs (EcircRNAs), which are generated by backsplicing and localized predominantly in the cytoplasm, Exon-intron circRNAs (EIciRNAs), which are circularized with intronic sequences retained between the backspliced exons and localized predominantly in the nucleus, and Intronic circRNAs (ciRNAs), which are derived from intronic lariat RNA precursors and localized in the nucleus. MecciRNAs are circRNAs encoded by the mitochondria, which are distributed in the mitochondria and in the cytosol.

angina, heart attack), strokes, hypertension, cardiomyopathy, arrhythmia, valvular diseases et al. [56,58]. A large amount of circRNAs have been identified in the hearts of humans, rats, and mice [59,60]. Lines of evidence have revealed the important roles of circRNAs in the pathophysiology of cardiovascular disease [61–89] (Fig. 2; Table 1).

3.1. Atherosclerosis and myocardial infarction

Atherosclerosis is a chronic disease in which plaque buildup on the inner wall of an artery leads to a narrowing of the artery [90]. Atherosclerosis could lead to heart attack and stroke with a high risk of death [90]. Pathogenic causes of atherosclerosis are complicated, and it is commonly accepted that aberrant proliferation of vascular smooth muscle cells (VSMCs) promotes plaque formation [91]. Increased low-density lipoprotein (LDL), hypertension, obesity, and diabetes will increase the morbidity of atherosclerosis [92]. Both promoting and inhibiting roles of circRNAs in the atherosclerosis process have been reported. circANRIL which is transcribed from the CVD risk locus on chromosome 9p21 has been reported as a suppressor of atherosclerosis. The expression of circANRIL is increased in peripheral blood mononuclear cells (PBMC) and plaque of coronary artery disease (CAD) patients with 9p21 atheroprotective genotype. CircANRIL binding to pescadillo homologue (PSE1), a key regulator of 60S ribosome biogenesis rRNA, prevents ribosomal RNA (rRNA) maturation, induces nucleolar stress, and eventually results in increased apoptosis and proliferation inhibition [63]. CircCHFR is reported aberrantly overexpressed in the oxidized-LDL (ox-LDL)-induced VSMCs. CircCHFR enhances Cyclin D1 expression by sponging miR-370 and increases transcription factor FOXO1 (a miR-370 target) levels. CircCFHR/miR-370/FOXO1/Cyclin D1 pathway promotes the proliferation and migration of VSMCs and facilitates atherosclerosis [64].

Myocardial infarction (MI), known as heart attack colloquially, is one of the leading causes of death worldwide [93]. MI is defined pathologically as cardiac muscle death due to prolonged ischemia [94]. CircRNAs have been well-studied in the pathophysiologic progression after infarction including infarct healing and cardiac remodeling [95]. Myocardial infarction-associated circular RNA (MICRA, also known as circZNF609) is identified as a prognostic biomarker for predicting left vehicle (LV) dysfunction after MI. Blood levels of MICRA are lower in MI patients compared to healthy volunteers. Patients with a lower level of MICRA are at higher risk of LV dysfunction [71]. CircFndc3b has been reported to promote cardiac repair after MI. CircFndc3b is significantly down-regulated in heart tissues from post-MI mice and ischemic cardiomyopathy patients. Intra-myocardial injection of circFndc3b-expressing AAV9 viral in post-MI mice enhances angiogenesis and improves cardiac functions after MI. Mechanistically, circFndc3b elevates the expression of vascular endothelial growth factor-A (VEGF-A) by interacting with fused in sarcoma (FUS) [72].

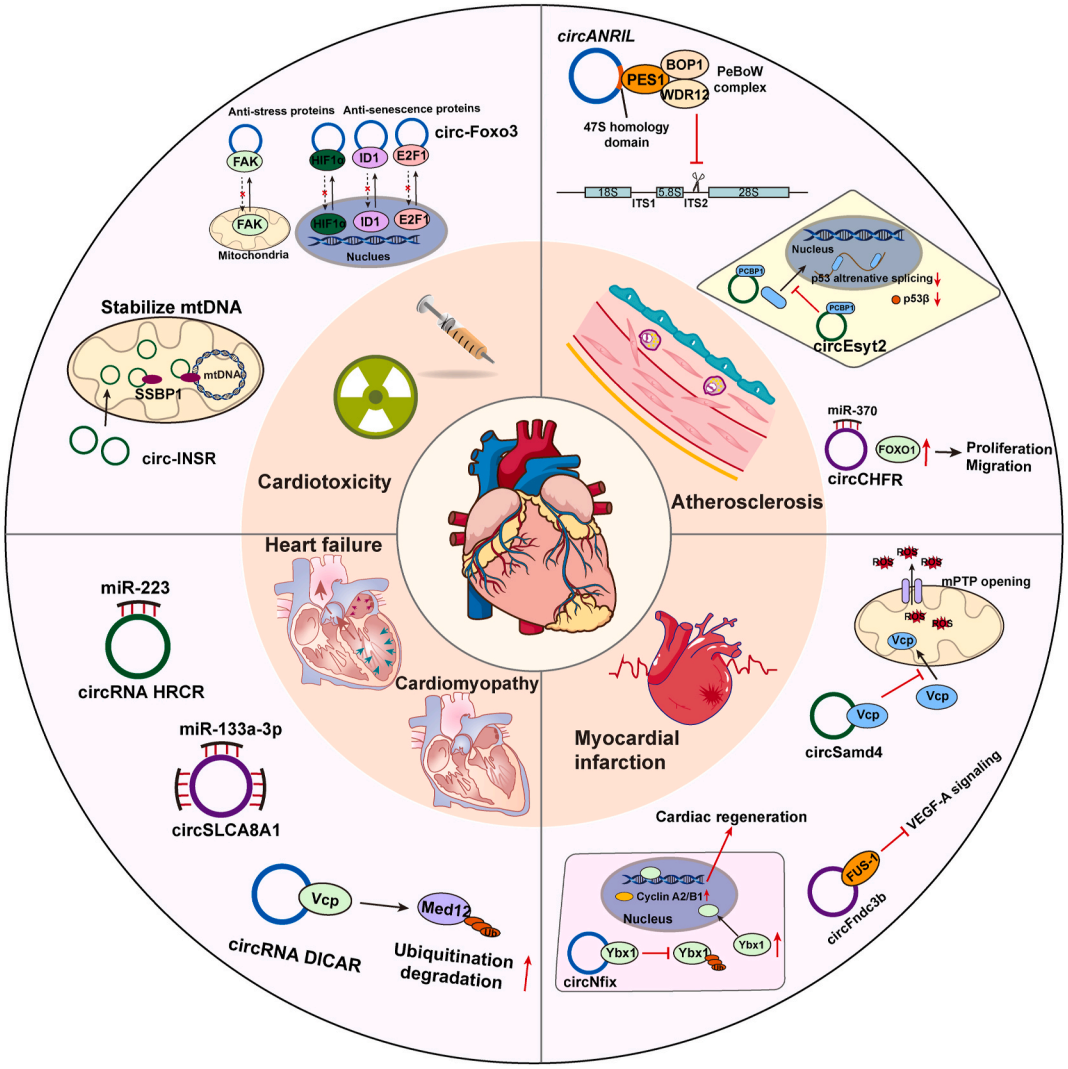

Fig. 2. Graphical demonstrates the representative circRNAs in cardiovascular diseases.

Table 1
circRNAs in cardiovascular disease.

circRNA	cardiovascular disease	Function	Molecular mechanism	Species	Ref
circANRIL	Atherosclerosis	Atheroprotective function through increasing apoptosis and inhibiting proliferation of vascular smooth muscle cells (VSMCs) in atherosclerotic plaques	Prevents ribosomal RNA (rRNA) maturation by binding to PSE1	Human Rat	[63]
circCHFR	Atherosclerosis	Promotes the proliferation and migration of VSMCs and facilitates atherosclerosis	Increases Cyclin D1 by sponging miR-370 and increasing FOXO1 levels.	Human	[64]
circRSF1	Atherosclerosis	Inhibits apoptosis and inflammation of human umbilical vein endothelial cells (HUVECs)	Increases HDAC1by sponging miR-135b-5p	Human	[65]
circRNA-0044073	Atherosclerosis	Increases the proliferation and invasion of VSMCs and HUVECs	Sponges miR-107 and activates the JAK/STAT pathway	Human	[66]
circRNA ZNF609	Vascular dysfunction	Promotes vascular dysfunction	Sponges miR-615-5p and increases MEF2A expression	Human Mouse	[67]
hsa_circ_0124644 hsa_circ_0098964	Coronary artery disease	Diagnostic biomarkers	Peripheral blood circRNAs	Human	[68]
hsa_circ_0001445	Coronary artery disease	Diagnostic biomarker	Plasma circRNA	Human	[69]
circEsyt2	Atherosclerosis, hypertension	Enhances Vascular remodeling	Binds to PCBP1 and regulating its intracellular localization	Mouse	[70]
circRNA MICRA (circZNF609)	Myocardial infarction (MI)	Prognostic biomarker for predicting left vehicle (LV) dysfunction after MI	Patients with a lower level of MICRA are at higher risk of LV dysfunction	Human	[71]
circFndc3b	MI	promote cardiac repair after MI	Elevates the expression of VEGF-A by interacting with FUS	Human Mouse	[72]
circNfix	MI	Loss of circNfix promotes angiogenesis and cardiomyocyte proliferation in mice	Represses the expression of cyclin A2 and cyclin B1 by promoting ubiquitination degradation of Ybx1	Human Mouse Rat	[73]
circSamd4	MI	Preserves LV functions in mice post-MI	Participates in antioxidant response in cardiomyocytes	Human Mouse	[74]
circPostn	MI	Promotes MI-induced myocardial injury and cardiac remodeling	Sponges miR-96-5p	Human Mouse	[75]
circRNA FEACR	MI	Suppressed MI and improves cardiac function	FEACR-NAMPT-Sirt1-FOXO1-FTH1 axis	Mouse	[76]
Cdr1as	MI	Promotes MI	Sponges miR-7a	Mouse	[77]
circSLCA8A1 (circNCX1)	Heart failure (HF)	Involves in many pathophysiological processes of heart diseases	Sponges miR-133a-3p	Human Mouse Rat	[78–82]
circRNA DICAR (mm9_circ_008009)	HF	Inhibit the pyroptosis in diabetic cardiomyopathy (DCM)	Mediates DICAR-VCP-Med12 degradation	Mouse	[83]
circSnx12	HF	Involves in ferroptosis during heart failure	Sponges miR-224-5p	Mouse	[84]
circRNA HRCR	HF	Inhibits cardiac hypertrophy and HF	Sponges miR-223	Mouse	[85]
circ-Foxo3	HF	Downregulated circ-Foxo3 attenuates doxorubicin (DOX)-induced cardiomyopathy	Regulates subcellular locations of ID1, E2F1, FAK, and HIF1a	Human Mouse	[86]
Circ-INSR	HF	Protective role against DOX-induced cardiotoxicity	Interacts with mitochondrial SSBP1, and stabilizes mitochondrial DNA	Human Mouse	[87]
circ-Amotl1	HF	Reduces DOX-induced cardiomyocyte death	Binds to AKT and activates its phosphorylation	Human Mouse	[88]
circITCH	HF	Alleviates DOX cardiotoxicity	Sponges miR-330-5p	Human Mouse	[89]

CircNfix is conserved among human, rat, and mouse, and is highly abundant in cardiomyocytes. CircNfix represses the expression of cyclin A2 and cyclin B1 by promoting ubiquitination degradation of Y-box binding protein 1 (Ybx1). Loss of circNfix promotes angiogenesis and cardiomyocyte proliferation in mice [73]. At the cellular level, mitochondrial reactive oxygen species (ROS) are generated during the MI, especially after reperfusion. Cardiomyocytes under oxidative stress and excessive ROS induces cardiomyocyte damage and even apoptosis [74]. Thus targeting mitochondria-derived ROS has become a novel therapeutic strategy [96]. Mitochondria-localized circSamd4, selectively expressed in fetal and neonatal cardiomyocytes, is found to participate in antioxidant response in cardiomyocytes. AAV9-mediated circSamd4 overexpression reduces ROS injury and preserves LV functions in mice post-MI [97].

3.2. Cardiomyopathy and heart failure

Cardiomyopathies are multi-cause diseases and frequently present as the syndrome of heart failure (HF) [98,99]. The main types of cardiomyopathies include dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), and arrhythmogenic right ventricular dysplasia (ARVC) [98,99]. DCM and HCM are the two most common types of cardiomyopathies. HCM is most often inherited and caused by pathogenic sarcomere variants, while DCM can be developed from genetic and non-genetic inducements [56]. A series of studies have demonstrated that circRNAs participate in cardiac fibrosis, cardiomyocyte functions, and cardiac functions during the development of cardiomyopathy. Hundreds of circRNAs are found dysregulated in heart tissues from DCM patients [78,81,82], indicating a potential functional role of circRNAs in DCM. CircSLCA8A1 (also known as circNCX1) is identified as a highly expressed circRNA in cardiomyocytes and is abnormally upregulated in DCM patients [78–82]. CircSLCA8A1 is multifunctional and involved in many pathophysiological processes of heart diseases [80]. Mouse circSlc8a1 functions as a microRNA sponge with 10 potential target sites for miR-133a which is a key regulator of cardiac hypertrophy [80,100]. Downregulation of circSlc8a1 attenuates pressure overload-induced cardiac hypertrophy in mice with transverse aortic constriction (TAC) [80]. CircSlc8a1-miR133a axis is implicated in regulating DCM as AAV9-mediated overexpression of circSlc8a1 *in vivo* or miR-133a deficient induces cardiac dilatation and heart failure [101]. Moreover, a potential role in ischemia injury of circSlc8a1 is reported, in which circSlc8a1 expression is increased by ROS response in rat cardiomyocyte cells and the elevated circSlc8a1 promotes cardiomyocyte apoptosis by sponging miR-133a-3p [79]. Collectively, an aberrantly high level of circSLCA8A1 seems to be related to diverse heart diseases, and

downregulating circSLCA8A1 might be a potential therapeutic strategy.

3.3. Anti-cancer therapy-induced cardiotoxicity

The incidence of cancer is rising globally, and the survival time of patients after diagnosis and treatment has increased due to the development of cancer therapy [102]. However, long-term cancer therapies unintentionally bring a series of cardiovascular complications [103–105]. Anthracyclines such as doxorubicin (DOX), are the most frequently mentioned cardiotoxic anti-cancer drugs [106,107]. The cardiotoxicity of DOX is extensively studied. DOX accumulates in the mitochondria and leads to cardiomyocyte dysfunction by increasing mitochondrial ROS. Excessive mitochondrial ROS induces the opening of the mitochondrial transient permittable pore (mPTP) and the sustained opening of mPTP leads to depolarization of the mitochondrial membrane. Cardiomyocytes with impaired mitochondria are subject to apoptosis [106–108]. Circ-Foxo3 is highly expressed in the aging hearts of mice and patients which induces cardiomyocyte senescence. Mechanistically, circ-Foxo3 interacts with proteins that are involved in cell senescence (ID1 and E2F1) and anti-stress response (FAK and HIF1a), and regulates their subcellular locations. Downregulating circ-Foxo3 attenuated DOX-induced cardiomyopathy in mice [109]. A protective function of circ-Amotl1 under DOX cardiotoxicity is also reported by the same research group as circ-Foxo3. circ-Amotl1 reduces Dox-induced cardiomyocyte death by binding to AKT and activating AKT phosphorylation [88]. MicroRNA sponge such as circITCH, alleviates DOX cardiotoxicity by sponging miR-330-5p [89]. Circ-INSR plays a protective role against DOX-induced cardiotoxicity by interacting with mitochondrial single strand binding protein 1 (SSBP1), stabilizing mitochondrial DNA (mtDNA). Circ-INSR is decreased in the hearts of DOX-treated patients and mice. Overexpression of circ-INSR via AAV9 viral in DOX-treated mice reverses DOX-induced cardiotoxicity [87].

Radiation treatment-induced cardiotoxicity is being increasingly concerned. Free radicals generated by radiation cause damage to the heart [105,110]. Radiation leads to endothelial injury and triggers an inflammatory response, raising the risk of atherosclerosis and myocardial ischemia [105]. Radiation also results in fibrosis of the myocardium and epicardium [105]. CircFOXO3 decreases apoptosis in cardiomyocyte cell lines under radiation, indicating a protective role under radiation-induced cardiotoxicity [86]. However, only a limited number of studies focus on circRNA in radiation-induced cardiotoxicity. Considering dysfunction of the heart itself being the final consequence, the circRNAs mentioned above may also serve as therapeutic targets in radiation-induced cardiac diseases.

4. Future directions and conclusion

CircRNAs are featured by their characteristics such as high stability, long half-life, low immunogenicity, translatability, tissue- and developmental stage-specificity, which renders circRNAs as promising therapeutic molecules in early prevention, diagnosis, clinical intervention, and even prediction of response to therapies of diseases including CVD. Despite significant progress in identifying and characterizing circRNAs, further studies are needed to address the crucial unknown factors and limitations in research investigating circRNAs' biological processes. A question in point is that circRNAs often exert their functions through one or multiple molecular mechanisms in many tissues and diseases, and the factors determining circRNA behaviors are multifactorial. Despite the vast majority of studies focused on the roles of circRNAs functioning as competitive endogenous RNAs in diseases including CVD, the actual number of circRNAs that are able to effectively contribute to disease progression by miRNA sponges is far lower than previously proposed [111,112]. miRNA binding sites of circRNAs and the capability to sponge miRNAs are not positively correlated, and some circRNAs with only one miRNA binding site could also function [112]. As many circRNAs are expressed with low levels, the ratio of circRNA/miRNA, as well as the relationship between the miRNA binding sites of circRNAs and the mRNA target sites of miRNAs, may be critically required for the target de-repression through ceRNA mechanism as most physiological changes in circRNA do not affect miRNA activity [111,113,114]. Necessary assessments of the accurate copy numbers and circRNA/-miRNA ratio under both physiological and pathological conditions in cells and tissues are fundamental to understanding circRNAs acting as miRNA sponges. Another frequently reported functional mechanism for circRNAs in CVD is circRNA-protein interactions, which refers to one circRNA may exclusively bind to a single protein or a complex of proteins under specific pathological conditions, or multiple circRNAs may form a circRNA-protein complex [115]. However, circRNAs seem to possess lower RBP binding density than their linear counterparts [116], which calls for novel and efficient algorithms for the assessment of potential circRNA binding. Additionally, circRNA functions in different diseases seem to vary with multiple modes, suggesting their contribution to a specific disease phenotype is likely to be context-dependent [117]. For example, circCcnb1 may exert distinct roles under different p53 backgrounds [118]; circCCAC1 plays dual roles in CCA cells and endothelial monolayer cells by sponging miR-514a-5p and redistributing protein, respectively [119]. Appropriate controls and rescue experiments remain crucial to validate circRNA functions and to rule out the false positive effects. As progress in investigating circRNA functions was made mainly in tumor progression and repression, similar approaches of investigation can also be applied to extensive areas such as cardiovascular research and even panvascular diseases (PVD) [120].

Several approaches have been developed to modulate circRNA levels and target circRNAs for therapeutic applications. Specific siRNAs or shRNAs that target the back-splicing junctions of circRNAs, coupled with lipid-based polymers, are the most widely used circRNA knockdown strategies both *in vitro* and *in vivo*. CRISPR/Cas9-mediated editing systems have also been utilized in modulating circRNA levels. CRISPR/Cas9 system targets the flanking intronic complementary sequences of circularizing exons or gene loci to disrupt circRNA biogenesis [121]. For example, knockout of circNfix by CRISPR/Cas9 system within cardiomyocytes both *in vitro* and *in vivo*, which increased the proliferation of HL-1 cardiomyocytes leading to cardiac regeneration after MI [73]. Meanwhile, the CRISPR/Cas13 system, unlike CRISPR/Cas9, targets the back-splicing junctions of circRNAs and shows high specificity and efficiency of knockdown, with fewer effects on their corresponding linear mRNA [122]. *In vitro* or chemically synthesized circRNAs are also used to enhance circRNA expression, usually as miRNA sponges [123]. However, obstacles remain such as off-target gene silencing, nonspecific tissue or cell type targeting, toxicity of gold nanoparticles, synthetic circRNA immunogenicity, etc [124], manifesting a large gap between basic science and clinical practice.

Highly efficient delivery systems are key to circRNA-based therapeutics, as circRNAs cannot cross cell membranes by nature and are prone to sequester within endosomal compartments when internalized. Lipid nanoparticles (LNPs) are the most widely used nanocarrier for RNAs, including circRNAs [125]. Once endocytosed, LNPs destabilize the endosomal membrane and release circRNAs into the cytoplasm [126]. Lentiviral and adenoviral vectors are effective approaches used to deliver and overexpress circRNAs *in vivo*. For example, adeno-associated viruses (AAVs) were reported to deliver circRNAs in improving cardiovascular function in transverse aortic constriction (TAC) mice [127]. AAV9 vector-based overexpression of the conserved circITCH partly prevented doxorubicin-induced cardiotoxicity in mice [89]. Additionally, *in vitro* synthesized circRNAs combined with nanoparticle delivery facilitate bioimaging and development for therapeutic drugs [128,129]. Exosomes from various sources are delivery vehicles for RNA transport and RNA level modulation [124,130]. One of the advantages of exosome-mediated delivery is exosomes protect RNAs from degradation and promote cellular uptake without triggering immune responses, which provides additional options for future *in vivo* studies [131].

Some circular RNAs have been uncovered with protein-coding

capacity [55,132,133]. An engineered circRNA with an internal ribosomal entry site (IRES) can be translated *in vivo* [55,132]. Due to the covalently closed ring structure, circRNAs can be resistant to exonuclease and are more stable than linear RNAs in cells and human fluid [21]. A longer half-life time allows circRNA to contiguously produce more significant amounts of proteins [134]. Circular RNA vaccines against SARS-CoV-2 have demonstrated effective protection in mice and monkeys [135]. This breakthrough provides a new insight into CVDs medicine design that engineering a circRNA translation tool expressing pharmaceutical proteins in patients.

The development of specific and effective approaches is key to the future of circRNA-based therapeutics. Silencing of an oncogenic circRNA or overexpression of circRNAs that protect from diseases provides therapeutic strategies for clinical treatments. Druggable circRNAs that rely on *in vitro* or chemical synthesis are also promising molecules for drug design, despite this approach is often limited by the production of circRNAs on a large scale. Future investigations are expected to focus on the safety and efficacy of nanoparticles and exosomes. More practical approaches that target or deliver circRNAs *in vivo* would promote the therapeutic use of circRNA-based therapeutics.

CVD is responsible for morbidity and mortality worldwide, with an increasing focus on efficient early diagnosis and targeted therapy. Despite the recent advances in research, novel diagnostic approaches and therapeutic interventions are still needed for clinical management. The landscape of circRNA expression in the human heart has been investigated in which the full spectrum of cardiac circRNA expression corresponding to their cognate cardiac-expressed linear protein-coding and non-coding genes were demonstrated with a high-abundance of specific cardiac-expressed circRNA was revealed [59]. Considering the complexity of cardiovascular research as compared to cancer research, experimental approaches and animal models need to be optimized. For example, the use of heart tissue and the crucial function of the individual make cardiovascular research not easily accessible. Despite the potential of circRNA as non-invasive CVD biomarkers is enormous, a huge amount of bioinformatics data with experimental validation are required before clinical trials. More detailed experiments with patient samples, such as biofluid, and exosomes, in the context of clinical research should be implemented corroborating with preliminary animal models. Furthermore, AI-assisted diagnosis with other high-quality data helps identify shared and unique characteristics in various pathological contexts, especially for early detection of CVD. Some disease-related databases such as circRNADisease v2.0 are powerful tools for predicting the associations of circRNAs and diseases [136]. Evolutionarily conserved circRNAs are gaining increasing attention [137–139]. Of note, a comparison of human and mouse circRNA datasets showed that 15% of circRNAs are conserved splice sites in orthologues genes, and as much as 10% of circRNAs are evolutionarily conserved in human, mouse and rat hearts [60,140,141], which facilitates the translational research from animal model discoveries into human CVD research. These CVD-associated circRNAs, together with exosome circRNAs easily obtained from serum collected, are therefore believed to be pioneers in paving the road for drug development.

CRediT authorship contribution statement

Xu Liu: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Xuelin Yao:** Writing – review & editing, Writing – original draft, Methodology. **Liang Chen:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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