Biogenesis of circular RNAs and their roles in cardiovascular development and pathology

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Introduction

With the development of RNA sequencing (RNA-Seq) technology, a new type of RNA named circular RNA (circRNA) has been identified in many organisms including yeasts, plants, protists, fruit flies, worms, zebrafish, mice, rats and humans [1–8]. The eukaryotic circRNAs are covalently closed, single-stranded RNAs that lack the 5' caps and 3' poly-A structures. The ligated 5' and 3' ends of circRNAs make them resistant to exoribonuclease degradation, and therefore more stable than linear RNAs [9]. Numerous studies have demonstrated gene regulatory functions of circRNAs, especially in mammals [2,10,11]. It has also been reported that circRNAs play critical roles in the development and progression of diseases such as Alzheimer’s disease and cancer, providing the pathological molecular mechanisms and directing new strategies for disease treatment [12–21] and diagnosis [22,23].

The cardiovascular system is the ‘engine’ of the organism and cardiovascular disease is one of the fatal diseases in humans. To date, the molecular mechanisms of cardiovascular disease are still incompletely understood. Numerous circRNAs have been detected in the cardiovascular system, implying they may play considerable roles in cardiovascular development and disease. However, some basic questions need to be clarified: how are these circRNAs specifically generated in the cardiovascular system? What are the expression

Circular RNAs (circRNAs) are a newly discovered type of RNA generated by back-splicing of precursor mRNA and found in many species. They are, expressed in a tissue-specific manner and fulfill regulatory activities in many biological processes. Recent research has revealed that circRNAs play critical roles in the development and pathologies of the cardiovascular system. Some of these circRNAs show aberrant expression and regulatory activities during heart disease including heart failure and cardiac infarction and hypertrophy. These findings suggest that circRNAs might be a suitable target for the treatment and prevention of heart disease. In this review, we summarize the latest research on the biogenesis and functions of circRNAs with emphasis on the regulatory roles of circRNAs in the development and pathologies of the cardiovascular system.

Abbreviations
ANRIL, antisense non-coding RNA in the INK4 locus; circRNA, circular RNA; cIRNA, circular intronic RNA; DCM, dilated cardiomyopathy; ecircRNA, exonic circular RNA; ecircRNA, exon–intron circular RNA; HCM, hypertrophic cardiomyopathy; HRCR, heart-related circRNA; IRES, internal ribosome entry site; IncRNA, long non-coding RNA; m6A, N6-methyladenosine; MBL, muscleblind; MI, myocardial infarction; miRNA, micro RNA; ORF, open reading frame; Pol II, polymerase II; pre-mRNA, precursor mRNA; QKI, Quaking; RBM20, RNA binding motif protein 20; RBP, RNA-binding protein; RNA-Seq, RNA sequencing; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein.
profiles of circRNAs in cardiovascular development and disease? And how do circRNAs contribute to cardiovascular development and disease? Therefore, the focus of this review is to delineate the discovery, biogenesis, expression and functions of circRNAs in cardiovascular development and disease.

**Discovery of circRNAs: from worst by-products to worthwhile regulators**

CircRNAs in eukaryotic cells were initially discovered by Hers and Coca-Prados [24]. By means of electron micrographs, they observed RNAs with circular structures in the cytoplasm of HeLa cells, but this study did not include any sequencing analysis. In the 1990s, several circRNAs were successively identified. Nigro et al. [25] first reported four novel types of human tumor suppressor gene (dcc) transcripts. PCR-amplification and sequencing showed that exons were connected in an abnormal order, suggesting that these exons were covalently linked in circular structures. In later years, the circular transcripts of human ets-1 [26], human mll [27], mouse fmm [28], sry in mouse testis [29], human cytochrome P450, 2C18 and 2C14 genes [30,31], rat shbg [31], human dystrophin [32], monkey slc8a1 [33], fruit fly mbi [34], a human non-coding RNA named antisense non-coding RNA in the INK4 locus (ANRIL) [35], and the antisense circular transcript of cdr1 [36] were subsequently identified. Although the above-mentioned research revealed the presence of circRNAs, for the past two decades these were considered as non-functional accidental by-products of aberrant splicing. The development of RNA-Seq technology thoroughly overthrew this viewpoint. Since 2012, numerous circRNAs have been identified in human, mouse, worm and fruit fly tissues [1,2,5], showing that circRNAs are widespread and stage- or cell type-specifically expressed. Jeck et al. [1] identified more than 25 000 circRNAs in human fibroblasts and 69 orthologues of human circRNAs in mouse testis. Memczak et al. [2] screened out 1950, 1903 and 724 circRNAs from the RNA-Seq data of human, mouse and Caenorhabditis elegans, respectively. Westholm et al. [5] annotated >2500 fruit fly circular RNA from diverse developmental stages, tissues and cultured cells. In addition, a substantial number of exonic circRNAs are identified in other species including yeasts (Schizosaccharomyces pombe and Saccharomyces cerevisiae), protists (Plasmodium falciparum and Dictyostelium discoideum) and plants (Arabidopsis thaliana and Oryza sativa) [37]. Notably, sequence analysis showed most of the circRNAs in human and fly contained micro RNA (miRNA) seed regions [2,5]. An antisense circular transcript of the human cdr1 gene, named CDR1as, harboured 63 conserved binding sites for the miRNA miR-7 and can inhibit the activity of miR-7 [2]. Hansen et al. reported that the circRNAs CDR1as and Sry could act as sponges for miR-7 and miR-138, respectively [10], revealing that circRNAs can regulate miRNAs. These studies have shown that circRNAs are a large class of RNAs with potential regulatory activities.

**Biogenesis of circular RNA: insight into tissue-specific expression and cellular localization**

In eukaryotes, precursor mRNAs (pre-mRNAs) are synthesized by RNA polymerase II (RNA Pol II). Pre-mRNAs then undergo spliceosomal splicing to generate linear mRNAs. Canonically, the 2’-OH of a branch point 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 initiates a nucleophilic attack on the 3’ splice site, joining the exons in the genomic order and releasing the intronic lariat [38]. CircRNAs are also generated from pre-mRNAs via canonical spliceosomal signals (the circularization efficiency being dependent on the presence of canonical splice sites bracketing the exons). However, the circularization is regulated by back-splicing, which is different from the canonical splicing. Although how exactly the spliceosome is involved in back-splicing remains unknown, it is reasonable to propose that in back-splicing, following the sequential assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) on the pre-mRNA, the branch point in the upstream intron will attack the 5’ splicing site in the downstream intron, forming a lariat containing the exons [39–41]. It was also suggested that cotranscriptional back-splicing was present [42]. Two possible reasons were proposed: firstly, nascent chromatin-bound circRNAs were found, and secondly, altering the cotranscriptional splicing ratio of Pol II changes the ratio of linear to circRNAs for individual genes [42]. It should be noted that chromatin-associated RNA is not precisely equivalent to nascent RNA, as transcripts with mature, polyadenylated 3’ ends were found in chromatin fractionations, and thus this remains to be further investigated. By alternative back splicing, the adjacent splicing donor and acceptor finally result in the release of different types of circular exons [exonic circular RNA (ecircRNA) or exons with retained introns [11] [exon–intron circRNA (ecircRNA; Fig. 1A]. In some cases, the intronic lariats could also
form circRNAs by canonical splicing [circular intronic RNA (ciRNA); Fig. 1B]. In this case, the presence of 7-nt GU-rich elements near the 5' splice site and C-rich element near the branch point are essential; this motif may not be recognized by the debraching enzyme and is therefore essential for an intron lariat to escape from debranching [43]. It was first suggested that back-spliced exons were associated with Alu elements within long flanking introns by Jeck et al. [1]. Alu elements belong to the short interspersed nuclear element family of repetitive elements, and with over 1 million insertions, they make up more than 10% of the human genome [44]. Intronic flanks adjacent to circularized exons were approximately two-fold more likely to contain an Alu repeat than non-circularized exons. Additionally, Alu elements taken from introns flanking circularized exons were significantly more likely to be complementary (in inverted orientation) than non-complementary. Circularized exons were six-fold more likely to contain complementary Alus than non-circularized exons [1]. It is known that Alu elements can be mutagenic to the host as they can act as splice acceptors, inhibit translation of mRNAs and cause genomic instability. The formation of Alu exons is suppressed by the nuclear ribonucleoprotein HNRNPC, which acts by competing with U2AF65 for binding to the antisense Alu elements and thereby prevents the recognition of cryptic 3' splice sites [45]. A human long non-coding RNA (IncRNA) named 5S-OT was shown to regulate alternative splicing of multiple genes via RNA–RNA complementation mediated by the antisense Alu sequences at the 3' end and by interacting with the splicing factor U2AF65 [46]. It was also possibly involved in the biogenesis of circRNAs. Other research showed that nuclear RNA helicase DHX9 binds specifically to inverted-repeat
Alu elements. Loss of DHX9 leads to an increase in the number of circular-RNA-producing genes and the amount of circular RNAs produced [47]. Zhang et al. [11] computed the transcripts from H9 human embryonic stem cells and further showed that not only Alu elements but also non-repetitive complementary sequences in the flanking introns of circularized exons could promote circRNA formation. This was further verified in C. elegans and for a few circRNAs in Drosophila melanogaster [48,49]. They also suggested that competition between the complementary Alu elements regulated the alternative back-splicing. However, Alu elements are primate specific and most circularized exons in Drosophila were flanked with introns without complementary base pairs [49]. In addition, dominant alternative back-splicing sites are highly diverse among cell lines [50]. These data suggested the possibility of other regulatory mechanisms in back-splicing. Intriguingly, several studies demonstrated RNA-binding proteins (RBPs) are also involved in the biogenesis of circRNAs. Muscleblind (MBL) was the first RBP to be identified in such studies. In humans and flies, the second exon of the mbl gene, which is flanked by introns containing highly conserved MBL binding motifs, could be circularized as circMbl. Exogenous MBL stimulated the circMbl production by bridging the flanking introns. Conversely, mutation of the MBL binding sites significantly decreased circMbl production [39]. Conn et al. [51] observed that many circRNAs were regulated by Quaking (QKI) during the epithelial-mesenchymal transition in humans. The expression of QKI correlated with circRNA expression, and silencing of QKI markedly inhibited circRNA biogenesis. Conversely, inserting the QKI-binding motifs into the flanking introns increased the abundance of circRNAs. Double-stranded RNA-specific adenosine deaminase (ADAR) exhibits adenosine-to-inosine editing activity and was shown to reduce back-splicing by weakening the complementation of the adenosine-rich flanking introns [48]. In D. melanogaster, circularization of laccase2 gene exons was regulated not only by intronic repeats but also by heterogeneous nuclear ribonucleoprotein and SR proteins [49]. These results demonstrated that RBPs play regulatory roles in back-splicing, perhaps the same as in alternative back-splicing.

RNA-Seq revealed that the expression of circRNAs is cell/tissue and developmental stage specific. This was observed in a human fibroblast cell line (Hs68), Hela cells, human embryonic stem cells, cancer cell lines, leukemia cells, human malignant bone marrow, mouse brain and testis, and different worm developmental stages [1,52,53]. Whether the specific expression of circRNAs was regulated at the transcriptional or post-transcriptional level is still unclear. Generally, there is a positive correlation between circular and linear RNA expression levels. Although the abundance of a number of cell type-specific circular transcripts was disproportionately higher than their linear counterparts, it was attributed to the stability of circRNAs resulting in their accumulation in cells [54]. However, there are a few exceptions. For example, linear mRNA of the BNC2 gene was predominantly expressed in human myoblasts in growth conditions and down-regulated in differentiated myoblasts, while the expression level of circRNA of the BNC2 gene was reversed relative to the mRNA [55]. This indicated that the expression of circRNAs was not transcriptionally regulated, but controlled post-transcriptionally. It has also been reported that silencing of the heart-specific splicing regulator RNA binding motif protein 20 (RBM20) causes the dysregulation of circRNAs transcribed from the titin gene [56]. So, we can conclude that cell/tissue or developmentally specific RBPs are also involved in the regulation of circRNA production.

At the cellular level, the majority of the ecircRNAs are located in the cytoplasm [1,52], while ecircRNAs and ciRNAs are mainly concentrated in the nucleus [43,57]. It is known that linear mRNAs are exported to the cytoplasm while most linear RNAs containing introns are restricted to the nucleus [58,59]. The transport of linear RNAs is regulated by RBPs such as heterogeneous nuclear ribonucleoproteins and SR proteins [60]. Since RBPs also participate in circRNA biogenesis, we can hypothesize that the mechanism of circRNA export and transport might be similar to linear RNAs; however, the detailed mechanisms of transport of circRNAs still need to be fully investigated. In conclusion, multiple factors are involved in the biogenesis of circRNAs, while RBPs are most likely involved in the regulation of their expression and transport.

Putative functions: the core connected genes, proteins and miRNAs

circRNAs can be translated!

As the initiation of eukaryotic translation relies on the cap structure in the 5' end of mRNA and the recognition of the 5' cap is required for the assembly of the translation initiation complex [61], the cap structure-lacking circRNAs were always characterized as non-coding RNAs. Although an artificial circRNA inserted in an internal ribosome entry site (IRES) and open reading frame (ORF) could produce peptide [61],
whether the added IRES and ORF give the circRNA protein-coding ability is unknown. Recently, protein coding circRNAs, circZNF609 in human muscle cells [55] and circMbl in fruit fly head [62], have been reported. These circRNAs interact with the ribosome and are translated both in vitro and in vivo in a cap-independent manner (Fig. 2A). Wang et al. [63] also suggested a mechanism of cap-independent translation: N6-methyladenosine (m6A) modifications near the start codon of circRNAs can act as IRESs and affect translation initiation. The m6A can be recognized by YTH domain family protein 3, which recruits translational factors eIF4G2 and eIF3A to initiate translation. The m6A modification is synthesized by methyltransferase-like 3 and 14, while reversely demethylated by fat mass and obesity-associated protein. The abundance of the protein coded by circRNAs is very low, possibly because of the low translation initiation efficiency of cap-independent translation. It’s still unknown if these proteins are functional. Since cap-independent translation responds to different types of cellular stress, it may function in some developmental and pathological conditions.

### miRNA sponges

MicroRNA levels can be regulated by the binding of the miRNA to its complementary endogenous mRNA. CircRNAs are abundant, stable, specifically expressed in tissues and development stages, and enriched in conserved nucleotides. Therefore, it has been proposed that circRNAs compete with other RNAs for binding miRNAs (Fig. 2B). CDR1as, the antisense circular transcript of the cdr1 gene, was the first reported functional circRNA [2,10]. Its high expression has been reported in the brain and it acts as a negative regulator of miR-7. The 63 conserved miR-7 binding sites allow CDR1as to interact effectively with miR-7 and reduce miR-7 activity. The knock-down of miR-7 and over-expression of CDR1as both caused defects in zebrafish midbrain [2]. Sry, also a previously reported circRNA, contains 16 putative miR-138 target sites that serve as miR-137 sponges [10]. The long, partially repeated sequence of these two circRNAs facilitates the enrichment of miRNA target sites. As reported by Memczak et al. [2], other circRNAs contained at least one miRNA seed region, but whether these circRNAs could generally function as miRNA sponges like CDR1as and Sry is yet to be demonstrated. Recently, Zheng et al. [64] indicated that circHIPK3, a circRNA encoded by hipk3, could significantly inhibit miR-124, although it only harbored two miR-124 target sites. It is possible that the miRNA-sponge ability not only depends on the numerous miRNA target sites but also on the expression level of the circRNA itself and the affinity of the single miRNA target site. Overall, these results indicate that circRNAs may act as miRNA sponges and this potential function of circRNAs needs to be explored further in future studies.

### Protein binding

As circRNAs are enriched in the conserved genomic regions, which mainly harbor protein binding sites, it is highly possible that circRNAs are also able to act as protein sponges (Fig. 2C). CircRNAs interact with RBPs to function in transcription, post-transcription and translation. circMbl in fruit fly and human interacts with MBL to suppress the circularization of mbl exons [39]; circPABPN1 identified in HeLa cell can target HuR to affect transcription of polyadenylate-binding nuclear protein (PABPN1) mRNA [65]; the antisense circRNA ANRIL negatively mediates the pre-rRNA processing and ribosome biogenesis by binding pescadillo homolog 1 [66]. Surprisingly, a number of circRNAs have been reported to interact with various proteins without typical RNA affinity. Du et al. [67,68] demonstrated that a circRNA of foxo3 could bind a series of cellular stress factors involved in senescence, apoptosis and the cell cycle. The mechanism of interaction of circRNAs and none-RBP proteins is still unclear, and needs to be studied further.

### Transcriptional regulation of host genes

The nuclear-localized ciRNAs specifically act as the transcriptional regulators of host genes. The expression of host genes can be promoted by interaction of ciRNAs with Pol II complexes; for instance, depletion of ci-ankrd52 and ci-sirt7 decreases the transcription levels of ankrd52 and sirt7 genes, indicating their transcription-promoting activities [43]. icircRNAs remain in the nucleus and localize to the promoter of their host genes. They interact with the U1 snRNA–U1A–U1C complex and associate with Pol II to enhance the transcription efficiency (Fig. 2D) [69]. For instance, icircRNAs ElciEIF3 and ElciPAIP2 were found to associate with Pol II by immunoprecipitation. Further investigation showed these icircRNAs increased transcription by interacting with U1 snRNP and the promoters of the host genes (Fig. 2D). These interactions likely involve the U1 snRNA binding sites in the circRNA introns [57]. Although these intronic circRNAs regulate the transcription of host genes, whether the transcription of
other genes can be regulated by ciRNAs or eicircRNAs is still unknown.

**CircRNAs in the cardiovascular system**

CircRNAs are abundant in heart

CircRNAs are spatiotemporally expressed regulators during postnatal development and their pathophysiological roles have been reported in diseases such as cancer and neurological diseases. Cardiovascular disease is the most significant health problem worldwide and the leading cause of death, but the molecular mechanisms are still not completely uncovered. By means of RNA-Seq studies, large amounts of circRNAs were identified in cardiac tissues in human, rat and mouse [70,71]. The expression profiles showed that some candidates were differentially expressed (> 2-fold) in heart during pathological conditions (e.g. transverse aortic constriction, failing, ischemia, cardiomyopathy) and developmental stages (embryonic, neonatal and adult), suggesting that these circRNAs might be involved in apoptosis, necrosis, proliferation, regeneration and so on. Although cellular distribution and AGO2 immunoprecipitation sequencing (to test the affinity with miRNAs) have been demonstrated, the functional significance and upstream regulatory mechanisms of most circRNAs in heart development and disease are unknown. Recent emerging studies have shown how circRNAs play a role in development and disease in the cardiovascular system and are comprehensively described in the following sections.
CircRNAs in heart development

Heart development is a complex process involving alternative splicing of genes in the regulatory network [72–74]. Back-splicing events have also been reported during human fetal heart development [75]. During the 10th and 20th weeks of fetal development, global induction of circRNAs has been observed in the heart over time. Among the abundant circRNAs, two encoded by the slc8a1 (ncx1) and rhobtb3 genes, respectively, had the highest expression level and induction in the heart. This study further reported that these two circRNAs were also induced during in vitro directed differentiation of human embryonic stem cells to cardiomyocytes. The findings of Werfel et al. [70] revealed that circRNAs were differentially expressed between heart tissues of neonatal rats and adult rats. Data showed that the homolog of human circular transcripts of slc8a1 was enriched more than three-fold in neonatal heart over the adult heart, suggesting that circRNA of slc8a1 possesses a conserved function in heart development. It is worth mentioning that SLC8a1 is a sodium/calcium exchanger that is required for heart development and heart contractions [76]. RHOBTB3, a Rab9-regulated ATPase required for endosome to Golgi transport, was reported to be highly expressed in cardiac tissues [77]. However, the induction of these circRNAs was significantly higher than mRNA. It seems that the expressions of these heart-related genes’ circular transcripts (including other circRNAs, e.g. circASXL1, circFAT3 and circSMARCA5) in fetal heart development were post-transcriptionally regulated and uncorrelated with their linear isoforms. As mentioned above, some heart-specific RBPs are possibly involved in the regulation of circRNA expression; for example the heart enriched RBM20 correlates with the biogenesis of circRNAs of the titin gene [56]. Previous studies have demonstrated that RBPs are essential for heart development [78], and therefore we can speculate that, at least partially, RBPs facilitate heart development by regulating circRNAs. The exact regulatory relationship between individual RBP and circRNAs needs to be further explored. However, activities of circRNAs evidenced above have clarified that circRNAs were not simply splicing by-products in heart development but may play critical roles independent of host genes.

CircRNAs in vascular development

CircRNAs are not only involved in cardiac development but also reported to be involved in vascular development. A circRNA named cZNF292 was shown to be expressed in several vascular endothelial cells including human umbilical vein endothelial cells, microvascular and aortic endothelial cells [79]. The study also reported that the circRNA cZNF292 was highly induced under hypoxia, which is the key stimulus for angiogenesis. Silencing of cZNF292 in vitro inhibited the formation of tube and spheroid sprouting of endothelial cells, indicating that cZNF292 had a pro-angiogenesis function. Other circRNAs differentially expressed under hypoxia such as cAFF1 have also been reported, but further functional studies are required.

CircRNAs in myocardial infarction

To date, cardiovascular disease is the leading cause of death in humans. Among all types of cardiovascular diseases, myocardial infarction (MI) is responsible for the highest mortality. Generally, MI arises from occlusion of the coronary artery. The continuous ischemia leads to the death of cardiomyocytes, resulting in pathological left ventricle remodeling, cardiac dysfunction and ultimately heart failure. Therefore, attenuating cardiomyocyte death is the main strategy for MI treatment. In recent years, non-coding RNAs including miRNAs and lncRNAs involved in cardiomyocyte death has become an active research area. As a new type of non-coding RNA, circRNAs were also reported to be involved in cardiomyocyte death and MI. Wu et al. [80] performed microarray assay to detect the expression level of 1163 circRNA candidates in normal murine myocardial tissues and myocardial tissues failing due to MI. A total of 63 differentially expressed circRNAs were screened out, including 29 up-regulated and 34 down-regulated circRNAs. Furthermore, the authors validated the expression of 12 circRNAs by real-time PCR, suggesting these circRNAs might play critical roles in MI. It is worth mentioning that this study also reported two-fold up-regulation of the well-studied circRNA CDR1as. Simultaneously, another study by Geng et al. [81] reported the mechanism of MI injury-promoting activity of CDR1as. Consistent with the results of Wu et al. [80], it was shown that CDR1as was up-regulated in infarcted cardiac tissues and cardiomyocytes under hypoxia. Overexpression of CDR1as could promote apoptosis of cardiomyocytes induced by hypoxia and MI injury. Another study revealed that CDR1as acted as a myocardial pro-apoptotic factor by serving as an miR-7a sponge, while miR-7a could protect cardiomyocytes against MI-induced apoptosis by targeting the pro-apoptotic genes parp and spl1. This is the first functional research of circRNAs in MI.
Deng et al. [82] reported that a circRNA designated circRNA_081881 may be correlated with MI since it was down-regulated more than 10-fold in MI blood samples. Bioinformatic prediction showed that it could bind miR-548, which targets the mRNA of the MI-protective gene ppary. In addition, silencing of circRNA_081881 could suppress the expression of ppary, suggesting that circRNA_081881 might be a therapeutic target for MI. Although the interaction between circRNA_081881 and miR-548 and the MI-protective effect of circRNA_081881 need to be further certified, the significant decrease of circRNA_081881 in MI blood is useful for MI diagnosis.

**CircRNAs in cardiomyopathy**

Cardiomyopathies are heart diseases that affect the myocardium. Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are two types of high incidence cardiomyopathies. In HCM, the myocardium of the left ventricle is thickened and has a disrupted alignment, resulting in reduced blood flow, disabling cardiac functions, heart failure and even sudden cardiac death. In DCM, left or right ventricular hypertrophy leads to impaired ventricle systolic pump function and ventricular dilation. The detailed molecular mechanisms of cardiomyopathy are still, however, not completely understood. Two recent studies of circRNAs involved in cardiomyopathy might take us in a new research direction. Heart-related circRNA (HRCR), which had protective activities in myocardial hypertrophy and heart failure, was reported by Wang et al. [83]. The expression of HRCR was significantly down-regulated in the heart samples of mice infused with isoproterenol and subject to transverse aortic constriction. According to bioinformatic prediction, HRCR contained six putative target sites of heart failure-related miR-223. Coimmunoprecipitation and AGO2 immunoprecipitation assays demonstrated that HRCR could interact with miR-223 and retain it in the cytoplasm. The pro-hypertrophy activity of miR-223 could be attenuated by over-expression of HRCR and aggravated by silencing HRCR. Khan et al. [56] observed that abnormal circRNA splicing happened during cardiomyopathy. According to RNA-Seq, they detected a total of 826 back-splicing junctions from heart samples of both HCM and DCM patients. RT-PCR assay showed that 10 circRNAs of camk2d and titin genes correlated with cardiomyopathy. The expression of calcium/calmodulin-dependent protein kinase type II delta chain (CAMK2D) circRNAs was reduced in both HCM and DCM, while the expression of titin circRNAs was mainly reduced in DCM. Further study showed that the RBP RBM20 was essential for the production of titin circRNAs as the knock-out of RBM20 led to defects in the biogenesis of titin circRNAs. It has been known that RBM20 is required for splicing of cardiac-related genes such as titin and dysfunction of RBM20 causes DCM. This result supplemented the pathological mechanism involving RBM20 in HCM.

Diabetes mellitus is able to cause diabetic cardiomyopathy and one of the most serious pathologies of this is cardiac fibrosis. CircRNA_000203, a circRNA transcribed from the mouse myo9a gene, was shown to be associated with cardiac fibrosis [84]. circRNA_000203 is upregulated in diabetic mouse myocardium and angiotensin II-induced cardiac fibroblasts. It interacts with the anti-fibrotic miR-26b-5p to promote the expression of collagen, type I, α 2 (Col1a2) and connective tissue growth factor, enhancing the proliferation of cardiac fibroblasts. These studies indicated that circRNAs are also involved in cardiomyopathy by mediating the cardiac fibrosis. Collectively, the above reports provided clues for therapeutic targets of cardiomyopathy treatment.

**CircRNAs in cardiac senescence**

Mammalian cardiomyocytes are terminally differentiated. The lifespan of a cardiomyocyte is roughly equal to that of the individual. Inhibiting senescence of cardiomyocytes is significant for extending the lifespan of human beings. It was reported that a circRNA of the foxo3 gene named circFoxo3 could promote cardiac senescence [67]. The study also indicated that circFoxo3 was highly expressed in the heart tissue of aged individuals. Exogenous circFoxo3 could aggravate the heart senescence and doxorubicin-induced cardiomyopathy, while the silencing the endogenous circFoxo3 could suppress the symptoms. Mechanistically, circFoxo3 binds inhibitor of differentiation 1, transcription factor E2F1, focal adhesion kinase and hypoxia-inducible factor 1-α and maintains them in the cytoplasm, subsequently inducing cellular stress.

**CircRNAs in atherosclerosis**

Atherosclerosis is one of the most lethal diseases worldwide. It is the predominant cause of heart infarction and other coronary heart diseases. Multiple genomic studies have shown that atherosclerosis is associated with the INK4/ARF locus on human chromosome 9p21, where several tumor suppressor genes (p16INK4a, p15INK4b and ARF) and the long non-coding RNA ANRIL are transcribed. Intriguingly, several
circular transcripts of ANRIL had been reported and their expression correlated with atherosclerosis risk [35]. The molecular mechanism of one circular ANRIL isoform has now been identified [66]. This circANRIL impaired the maturation of ribosomes by binding to the 60S-peribosomal assembly factor PES1, leading to nuclear stress and p53 activation. As a result, the proliferation of smooth muscle cells and macrophages was inhibited while apoptosis was induced, indicating circANRIL could protect from atherosclerosis.

**CircRNAs in diagnosis of cardiovascular disease**

Exosomes are vesicles which are secreted into the extracellular fluid by cells. CircRNAs were shown to be enriched in exosomes due to their high stability, and the abundance of circRNAs in exosomes positively correlated with their cellular expression [85]. CircRNAs have been reported functioning as biomarkers in some diseases including cancer (e.g. hsa_circ_0000190, which is downregulated in gastric cancer [86]), major depressive disorder (e.g. hsa_circRNA_103636, which is downregulated in major depressive disorder [16]) and multiple sclerosis (e.g. circ_0005402 and circ_0035560, which are downregulated in multiple sclerosis [87]). During cardiovascular disease, the expression of some circRNAs have been reported to be dysregulated, and therefore analysis of the circRNA levels in serum has become a new method for cardiovascular disease diagnosis. For instance, hsa_circ_0124644 can be used as a diagnostic biomarker of coronary artery disease because it shows an upregulation in the blood of coronary artery disease patients [88]; the circular RNA from exon 1 of zinc finger protein 609 named myocardial infarction-associated circular RNA (MICRA) was reported as a predictor of left ventricular dysfunction after acute MI [89]. The MICRA level in blood of MI patients was lower than healthy people; moreover, MI patients with relatively low level of MICRA were at high risk of left ventricular dysfunction. CircRNAs are therefore appropriate for cardiovascular disease diagnosis and more circRNAs will be identified as potential biomarkers in future studies. Correspondingly, the diagnosis techniques, not only limited to qRT-PCR, for quick and accurate circRNA detection would need to be validated.

**Perspectives**

By means of advanced RNA-Seq and bioinformatics technologies, we have gradually realized that circRNAs are stable, abundant, conserved, spatiotemporally expressed and versatile in function. The research involving circRNAs has rapidly accumulated. However, some questions including the mechanisms of back-splincing, cellular localization, tissue specific expression, protein binding and transcription complex interaction are still uncertain. In addition, although it was recently discovered that circRNAs can be translated, the function of the coded proteins needs to be further investigated.

The involvement of circRNAs in disease is one of the most active research areas now. The research achievements will include new therapeutic targets for disease treatment. Cardiovascular disease is one of the most fatal disease and it has remained the focus of medical research. Although the circRNA research effort in cardiovascular disease is relatively small in comparison to cancer, neuropathy, and other diseases, all the reported results reflected the predominant roles of circRNAs in cardiovascular disease (Table 1). Meanwhile, the abundance of highly expressed circRNAs in the cardiovascular system detected by RNA-Seq also indicates that circRNAs might be indispensable for normal cardiovascular functions and development. Once their expression is dysregulated, cardiac dysfunction and disease could be caused. So far, the mechanism of most of these circRNAs is unclear, but it is known that miRNAs are important regulators in the heart [90–92]. AGO2 immunoprecipitation sequencing has shown the strong miRNA-interaction ability of circRNAs in the heart [70]. Therefore, some of the circRNAs might control the regulatory activity of miRNAs according to the circRNA–miRNA–mRNA pathway. However, there are still circRNAs without any seed region of miRNAs. CircRNAs containing conserved motifs are possibly able to interact with proteins, and the ones without protein-interacting regions may still bind proteins. More importantly, the discovery of a protein-encoding

| CircRNA     | Stage or disease involved                  | Effect | References |
|-------------|-------------------------------------------|--------|------------|
| circSLC8a1  | Heart development                         | Unknown| [70]       |
| CDF1as      | Myocardial infarction                     | ↑      | [81]       |
| circRNA_081891 | Myocardial infarction                  | Unknown| [82]       |
| MICRA       | Myocardial infarction                     | Unknown| [89]       |
| HRCR        | Hypertrophic cardiomyopathy               | ↓      | [83]       |
| circTitin   | Dilated cardiomyopathy                    | Unknown| [56]       |
| circRNA_000203 | Cardiac fibrosis                       | ↑      | [84]       |
| circFoxo3   | Myocardial senescence                     | ↑      | [67]       |
| cZNF292     | Angiogenesis                              | ↑      | [79]       |
| circANRIL   | Atherosclerosis                           | ↓      | [66]       |
| hsa_circ_0124644 | Coronary artery disease              | Unknown| [88]       |
function of circRNAs leads us in a new direction of circRNAs research. The 5’ cap-independent translation of circRNAs showed low efficiency; therefore, the proteins encoded by circRNAs may be dispensable. However, as cap-independent translation can be induced by cellular stress, the translation of circRNAs may play an important role in diseases such as cardiovascular disease (e.g. ischemic cardiomyopathy, a condition where cardiomyocytes are starved of nutrients and oxygen). Moreover, since it has been reported that IncRNAs could encode functional micropeptides [93], the circRNAs with a short ORF should not be ignored. In addition, bioinformatics tools are also significant in detecting and identifying of circRNAs [94]. Predictive tools involving RNA interacting (e.g. RNAhybrid [95]) and protein binding (e.g. MEME, RBPMap, SEAMOTE, RNAcontext, CATRAPID and RPISEQ [96]) can be used in functional prediction for circRNAs. According to the potential binding partners, we can infer the roles of circRNAs in pathology. More accurate and systematic bioinformatics tools will directly bridge circRNAs and the corresponding diseases. On the other hand, factors affecting the dysregulation of circRNAs in cardiovascular disease, including RBPs’ post-transcriptional regulation, are still scarcely studied and focusing on this axis of circRNAs may elucidate their mechanism of action. Furthermore, circRNAs may act as ideal biomarkers in the diagnosis of cardiovascular disease; however, in this case, the diagnostic techniques for quick and accurate circRNA detection, not only limited to qRT-PCR, need to be validated.

In conclusion, although circRNAs in the cardiovascular system have not been studied frequently, the regulatory role of circRNAs in the cardiovascular system indicates that these molecules could be potential therapeutic targets in cardiovascular disease treatment and prevention.

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Disclosures

None declared.

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