Molecular Therapy
Nucleic Acids
Review

Rapid Development of Targeting circRNAs in Cardiovascular Diseases

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Circular RNAs (circRNAs) are circularized, single-stranded RNAs that are covalently linked. With their abundance in tissues and developmental stage-specific expression, circRNAs participate in a variety of physiological and pathological processes. In this review, we discuss the development of circRNAs used as biomarkers and therapeutic targets for cardiovascular diseases (CVDs), focusing on recent discoveries and applications of exosomal circRNAs that highlight opportunities and challenges. Some studies have identified a spectrum of circRNAs that are differentially expressed in CVDs, while other studies further manipulated specific circRNA expression and showed an ameliorated pathogenic state such as ischemic injury, hypertrophy, and cardiac fibrosis. Studies and applications of circRNAs are being rapidly developed. We expect to see clinical use of circRNAs as biomarkers and targets for disease treatment in the near future.

Biogenesis of Circular RNAs

Circular RNAs (circRNAs) are a group of transcripts that are covalently looped, single-strand RNAs lacking polarities and polyadenylated tails.[1] They are generated at a lower efficiency compared to canonical splicing of precursor (pre-)mRNA and are sorted into three types by different circularizing mechanisms, that is, exonic circRNAs, intronic circRNAs, and intron-retained circRNAs. Most circRNAs originate from exons of coding regions, and the rest are from 3′ UTR, 5′ UTR, introns, intergenic regions, and antisense RNAs.[2] With different forms of splicing, a single gene locus can produce various circRNAs through alternative back-splice site selection.[3] Although less efficiently transcribed than linear RNAs, the number of circRNAs is still substantial because of their stability and relatively long half-life.[4] Exonic circRNAs account for more than 80% of known circRNAs, but the mechanism of biogenesis is still not clear. The inchoate back-splicing mechanism depends on complementary intron matches, which exist widely in the exonic circRNA formation process.[5] In 2003, two models of exonic circRNA formation were proposed: "lariat-driven circularization," and "intron pairing-driven circularization."[6] It is commonly acknowledged that back-splicing occurs in reversed orientation that connects a downstream 3′ splice site to an upstream 5′ splice site to generate circRNAs in most instances.[3] Intronic circRNA formation is different from that of exonic circRNA in splicing mechanism, depending on GU-rich sequences near the 5′ splice site and C-rich sequences near the branch point. The two segments bind into a circle first during the back-splicing process. After that, exonic sequences and intronic sequences in the binding part are cut out by the specific spliceosome. Finally, the remaining introns are eventually pieced together to form mature circRNAs.[7,8] This process is sophisticatedly regulated by spliceosomes.

The RNA spliceosome is an RNA-based enzyme consisting of five small nuclear ribonucleoprotein particle (snRNP) subunits U1, U2, U5, and U4/U6. U1 and U2 first base pair with the 5′ splicing site and 3′ branching site of pre-mRNA, respectively, and then U4/U6/U5 tri-snRNP is recruited. U6/U2/U5 is the catalytically activated spliceosome complex to splice circRNAs from its linear precursors. These circRNAs are also regulated by various types of proteins such as RNA-binding proteins (RBPs) and quaking protein.[9]

The relatively long half-life is one of the most attractive features of circRNAs. Due to their resistance to exonuclease, circRNAs confer their existence for more than 48 h compared to less than 10 h of linear

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form RNAs.[10,11] Serum circRNAs have a much shorter half-life at 15 s, probably due to the presence of circulating endonuclease.[11]

Functions of circRNAs

Increasing studies have shown that circRNA is an important regulatory element at the transcription and post-transcription levels. It carries out such functions by acting as microRNA (miRNA) sponges and competing endogenous RNAs (ceRNAs), binding to RBPs, regulating transcription, and in some cases translation into proteins.[12]

miRNAs are a large class of 22-nt non-coding RNAs (ncRNAs) that regulate gene expression via direct base pairing to target sites within mRNAs at the post-transcriptional level.[13] circRNAs can compete for miRNA-binding sites and regulate relative mRNA expression, as the ceRNAs. The function of circRNAs as miRNA sponges can influence miRNA activities.[14] Hansen et al.[15] discovered that ciRS-7 (also named CDRIas) functions as an miR-7 inhibitor; more than 70 conventional miR-7 binding sites were located. Expression of ciRS-7 efficiently inhibits miR-7, resulting in reduced miR-7 activity and increased miR-7-targeted transcription level. The function of ciRS-7 is consistent with the miRNA sponge and ceRNA hypothesis.[16] According to recent research, ciRS-7 showed widespread expression in neuroblastomas and frequent expression in astrocytoma, renal cells, and lung carcinomas,[17] as well as reduced ciRS-7 expression in transformed neuronal cells. Due to the function of ciRS-7 and high expression of miR-7- and ciRS-7-stimulated RNA-induced silencing complexes (RISCs), ciRS-7 may play profound roles in diseases such as cancer.[15]

Recent studies have identified many circRNAs functioning as post-transcriptional regulators.[14] Some circRNAs have also been implicated in transcriptional or post-transcriptional gene regulation of their host genes. For example, the circRNA generated by CDR1 is speculated to promote the expression of CDR1 sense mRNA, but the mechanism of the regulation is still unknown.[18] Zhang et al.[7] showed that circRNAs could regulate the expression of parental genes. They found that circRNAs were abundant in the nucleus with little enrichment for miRNA target sites, and a lower expression of their parental genes was detected after knockdown of circRNAs. Furthermore, the translation of a tumorigenic gene Yap was discovered to be inhibited by its own circular transcript (circYap). The circYap binds Yap mRNA and the translation initiation proteins eIF4G and PABP, leading to interfering translation initiation. By suppressing parental gene translation, this novel mechanism of post-transcriptional regulation function of circRNA brings insightful ideas about tumor intervention.[19] Recently, a group of regulatory circRNAs, named exon-intron circRNAs (EIciRNAs), has been identified to play an important role in transcriptional regulation.[20] EIciRNAs, such as circEIF3J and circPAIP2, enhance the transcription of their parental genes by interacting with U1 snRNP and RNA polymerase II.[20] circRNA can also act as a scaffold for RBPs to modulate transcription, similar to some long non-coding RNAs.[21]

Similar to mRNAs, some circRNAs have open reading frames (ORFs) and can be translated into protein peptides. Perriman and Ares[22] reported that a circRNA containing an ORF of green fluorescent protein (GFP) could direct GFP expression in *Escherichia coli*. Subsequently, AbouHaidar et al.[23] uncovered a circRNA of the virusoid associated with rice yellow mottle virus that encodes for a 16-kDa protein. Wang and Wang[24] discovered a robust protein production in minigenes containing structured or unstructured introns, indicating that the circRNAs can indeed function as mRNAs to direct protein synthesis. Most recently, three studies discovered that circRNAs can be translated under a cap-independent pathway induced by some unique sequences (internal ribosome entry site [IRES] and RRACH) and N^6-methyladenosine (m6A).[25–27] Translating circRNAs may have a function in cellular responses during tumor progression.

Exosomal circRNAs

Besides direct interaction with cellular molecules, circRNAs can also be exported by exosomes and may function in cellular communications. Exosomes are extracellular vesicles that are released from almost all cell types. These 30- to 200-nm-diameter vesicles share a topological structure with cells. The precursor of exosomes, the multivesicular body, will fuse with the cell membrane and release luminal contents, including proteins, lipids, RNA, and DNA. RNA was first described as one of the cargoes of exosomes by Ratajczak et al.[28] in stem cells; Oct-4 mRNA is transferred to other cells and leads to increased Oct-4 levels in recipient cells. Once being released into the extracellular matrix, exosomes elicit responses in target cells via various routes, including fusion with plasma membrane, endocytosis, and binding on the cell surface.[29] Some exosomes are released into the blood circulation and reach cells in distant tissues.[30] This novel way of intercellular communication has drawn an explosion of interest, as it has been found to be a contributing factor to several diseases such as cancer.

In numerous physiological and pathological conditions, non-coding RNAs can be detected in exosomic forms in the bloodstream.[31] miRNAs are particularly effective in regulating target genes and are a major group of cargo found in exosomes.[32] miRNAs are found to be transferred from tumor cells to control proliferation, invasion, and metastasis. Drug-resistant phenotypes can even be transferred by exosomal miRNA.[33] Clinical measurement of circulating exosomal miRNAs has already been studied and shows promising potential of being a biomarker.[34] circRNAs, as an emerging type of non-coding RNAs, are also reported to be found in exosomes and can be transferred into target cells.[35] Many studies on exosomal circRNAs have already linked exosomal circRNAs with various cancer diagnoses.[36–38] However, for cardiovascular diseases (CVDs), the leading cause of death in the world, its connection with circRNAs is less studied and there are very few studies published about exosomal circRNAs and CVDs. Although the function of exosomal circRNAs is still not fully understood, several lines of evidence show that it is functionally active in different physiological process such as stress response. For example, exosomal circHIPK3 released from hypoxia-pretreated cardiomyocytes (CMs) can be transferred to cardiac microvascular epithelial cells. The transferred cells are more resistant to oxidation due to circHIPK3 sponging of miR-29a.[39] As circRNAs are found to be

enriched in exosomes, they can be collected by blood sampling. Researchers have tested that exosomal circRNA is quantifiable for cancer diagnosis and prognosis.[2] More than 1,000 exosomal circRNAs are found to be useful to discriminate patients with tumors and healthy individuals.[40] Such findings may expand to CVDs.

With exosomes being recognized as a novel intercellular communication pathway, some key findings confirmed that exosomes are playing a central role in CVDs. Analysis of exosomal cargo confirmed that cardiac cell communication via exosomes is altered in cardiac fibrosis.[41] Exosomes are increasingly recognized to be related to cardiac diseases, and more effort is needed to elucidate the role of exosomal circRNA.[42] Stage-dependent, tissue-specific expression and its abundance make circRNAs a great candidate for serving as new biomarkers, which has attracted extensive attention globally. With increasing research conducted to study the relationship between circRNA and CVDs, a review of the current development is necessary. In this review, the relationship between circRNAs and CVD is discussed, as well as the latest research progress. Understanding mechanisms of circRNA function is a stepping stone of novel biomarker development and therapeutic targets, while study of exosomal circRNA may inspire novel therapeutic methods and drug delivery routes.

Cardiopathy

CVDs are the top cause of death globally, with about 17.9 million deaths in 2016.[43] CVDs are disorders related to the heart and vasculature, including stroke, heart rhythm disorders, cardiac arrest, atherosclerosis, coronary heart diseases (CHDs), valvular diseases, and peripheral artery diseases, among others.[44] The three most abundant cell types in heart tissue are cardiac fibroblasts (CFs), cardiomyocytes, and endothelial cells, with CFs being the most predominant cell type.[45] The resulting heart failure (HF) from pathogenic cardiac cells is the main cause of death. The prevalence of cardiomyopathy has increased over time, while the survival rate of heart failure after myocardial infarction (MI) has improved due to secondary prevention therapies.[44]

The contraction of the heart and blood pumping function is carried out by cardiomyocytes, which represent about 85% of total heart mass. Disorders related to cardiomyocytes can lead to life-threatening cardiac dysfunction in multiple pathological settings. Although cardiomyocyte apoptosis is essential and observable in healthy hearts, dysregulation of the phenomenon is linked to a series of CVDs.[46] Heart failure is associated with the loss of cardiomyocytes. Due to the lack of ability to regenerate, treatments of heart failure are heavily reliant on transplantation. It is reported that the number of cardiomyocytes that re-enter the cell cycle to proliferate after injury is far from being able to repair the damage.[47] Current research is focusing on circumventing such obstacles by transplanting self-regenerative skeletal muscle myoblasts. Cardiac myoblasts are the precursors of cardiac myocytes, with well-established cell lines, including rat H9C2 cardiac myoblast cells.

Excessive CF activation and proliferation will lead to cardiac fibrosis, which is the major problem in almost all heart diseases. Naturally, CF activation will be induced after ischemic/hypertrophic insult.[48] Activated CFs, termed myofibroblasts, will differentially express ECM proteins as a reparative response.[49] Studies have shown that exosomes derived from CFs carry miR-21 and enhance cardiac myocyte hypertrophy in pressure-overloaded myocardium. The results are consistent when inhibition of miR-21 reduced cardiac hypertrophy and remodeling.[50] Studies have shown that exosomal miRNAs such as miR-208a, miR-29b, miR-455, miR-21, and miR-155 play important roles in CF paracrine signaling via exosomes.[50–53] Additionally, studies from Sun et al. elucidated that CF activation and proliferation can be regulated by cytoplasmic circLAS1L via the miR-125b/SFRP5 pathway.[54] These discoveries lead to the question whether exosomal circRNAs are also involved in CF activation and warrant further investigation. If exosomal circRNA is involved, novel therapeutic strategies could be developed by using artificial circRNA-enriched exosomes.

Although many insightful studies have been published about exosomal non-coding RNA in cardiac diseases, we are still far from identifying the whole spectrum of pathways of non-coding RNA effects. More research should be carried out in terms of biogenesis, regulation, and delivery of exosomal non-coding RNAs.

Therapeutic Status Quo of CVD

Current clinical strategies against CVD depend on the type and severity. Therapeutic prescription ranges from changes of lifestyle, pharmacological treatments, gene therapy, surgery, and transplant. Despite advancements in treatment strategies, mortality and morbidity remains substantial.[55]

Lifestyle changes, including exercise, healthy diet, quitting smoking, less alcohol consumption, and healthy body weight, are proposed for primary prevention of CVD.[56] Pharmaceuticals associated with multiple pathways in CVD have been identified and novel treatments are emerging. Use of beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and aldosterone antagonists showed prolonged survival of patients with HF and cardiac fibrosis.[55,57] Biomaterial-based approaches have preserved or improved cardiac function in MI patients.[57] There are also several ongoing clinical trials for novel biomaterials (hydrogels) and regenerative medicine (autologous stem-cell sheet).[58,59] Numerous ongoing trials of gene therapies incorporate vectors such as adenovirus, retrovirus, and plasmids to deliver small interfering RNA (siRNA), miRNA, and so forth.[60] In the US, the median waiting time for a heart transplant in 2017–2018 was 6.9 months, and a total of 3,440 heat transplants were performed in 2018. Although the number of transplants performed increased and the waiting time gradually decreased after 2014, rejection and the 10-year mortality rate remained high (27% of the recipients underwent acute rejection in the first year post-transplant in 2016–2017, and the 10-year mortality rate in 2018 was 38.2%).[61] In order to improve the patients' quality of life, more work is needed to improve diagnostic tools for early, reliable, and non-invasive CVD detection and therapeutic strategies. Non-coding RNA could be an outstanding candidate.

Table 1. Recent Identification of circRNAs in CVD

		circRNA Identification in Pathogenic Hearts					
Disease/Details		No. of circRNAs Downregulated	No. of circRNAs Upregulated	circRNAs and Originating Host Gene	Upstream Gene/ Regulatory Pathway	Downstream Gene/Regulatory athway	Ref.
Ventricular septal defect	embryonic heart tissue	3,072	3,162				69
Myocardial ischemia/reperfusion injury		119	66				70
Heart failure		219	447				71
	dilated cardiomyopathy induced						74
Atrial fibrillation (heart tissue)		199	537				73
Atrial fibrillation (left atrial appendages)		104	374	hsa_circ_0003965 from TMEM245	glucagon signaling pathway		74
Atrial fibrillation (right atrial appendages)		268	267	hsa_circ_0004270, hsa_circ_0000075, hsa_circ_0030254, hsa_circ_0007271, etc		focal adhesion pathway	74
Atrial fibrillation (both right and left appendages)		3	20	hsa_circ_0004270, hsa_circ_0000075, hsa_circ_0030254, and others		ECM receptor pathway	74
				hsa_circ_0000075, hsa_circ_0082096, and others		SMURF1, TGFB2, GDF7, ROCK1, IFNG, LTBP1, LEFTY2, BMP6, FMOD, and FBN1 genes, TGF-β signaling pathway	74
Atrial fibrillation (atrial tissue)		57	51			circRNA-miRNA network, further GO, KEGG analysis	75
Coronary heart disease		37	73			circRNA-miRNA network, further NHGRI GWAS catalog, GO, and Reactome	76

ECM, extracellular matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GWAS, genome-wide association study.

Identification of circRNA in CVD

With strong evidence emerging of circRNA's relationship to pathogenesis, scientists are putting more effort into research connecting circRNA and cardiopathy. Horizontal and vertical comparisons of circRNA expression in different types of cardiopathy are being conducted. A substantial number of circRNAs have been identified (Table 1) to be expressed differently for a variety of diseases, including CHD, atherosclerosis, myocardial fibrosis (MF), MI,[62] HF, hypertension, and aneurysm, among others.[63–65] circRNAs were also vertically studied, and their expression was reported as tissue- and stage-specific, indicating that circRNAs are also involved in cardiac cell differentiation.[66] Therefore, circRNAs have strong potential to be a biomarker of CVDs and signals that direct cell differentiation, and they may have important roles in future regenerative medicine.[67]

With the adeno-associated virus (AAV)-based circRNA transgene method, deep sequencing techniques, and the advanced data analysis method, thousands of circRNAs have been characterized from several tissues and organisms.[68] Liu et al.[69] identified 6,234 differentially expressed circRNAs, of which 3,162 circRNAs were overexpressed, and 3,072 circRNAs were underexpressed at fold change in embryonic heart tissue with ventricular septal defects. 185 significantly and differentially expressed circRNAs were reported by Ge et al.[70] in extracellular vesicles isolated from murine heart after ischemia/reperfusion (I/R) injury. Another study reported 447 upregulated and 219 downregulated circRNAs in HF patients.[71] Werfel et al.[72] sequenced rat, mouse, and human rRNA-depleted heart circRNA samples after treatment with RNase R. More than 9,000 circRNA candidates were detected, of which about 30% were conserved between mice and rats, and 10% were conserved among all three species. They found 40 back splices stemming from the titin (Ttn) genes, and several Ttn-derived circRNAs were specifically and strongly enriched in adult or neonatal rat hearts. Ttn gene transcripts undergo complex and developmentally controlled differential splicing, resulting in thousands of isoforms. Taken together with the evidence that circRNAs can influence mRNA processing by competing with linear splice, it appears possible that circRNAs could be facilitating the diversity of Ttn splicing. Furthermore, they observed increased circRNA expression in mouse and human failing hearts. Jiang et al.[73] compared three atrial fibrillation (AF) patients and three healthy heart tissues and discovered 537 upregulated and 199 downregulated circRNAs in AF patients. Within these differentially expressed circRNAs, eight

upregulated and two downregulated circRNAs such as hsa_circRNA_100612 were found to interact with known AF-related miRNAs, that is, hsa-miR-892a, hsa-miR-3149, hsa-miR-3171, hsa-miR-892a, and hsa-miR-133b. hsa-miR-113b could target genes KCNIP1, JPH2, and ADRB1. KCNPIP1 is a cytosolic voltage-gated K^+ channel-interacting protein and is related to cardiac conduction; JPH2 has an important role in sarcoplasmic reticulum Ca^{2+} handling and ryanodine receptor Ca^{2+} channels; and ADRB1 is a member of a superfamily of cell surface receptors and has been an effective target for beta-blocking medications. Zhang et al.[74] compared circRNA expression in normal and AF hearts. They revealed 20 upregulated and 3 downregulated circRNAs out of 14,215 candidates in AF atrial appendages. Further ceRNA network analysis revealed that hsa_circ_0000075 and hsa_circ_0082096 were involved in AF pathogenesis via the transforming growth factor β (TGF-β) signaling pathway, which is a well-recognized player in AF. Some other target genes were also highlighted in the ceRNA, including IFENG, DGF7, and EDN1. Hu et al.[75] reported 108 circRNAs that were differentially expressed in patients with rheumatic heart disease and persistent AF. Bioinformatics study of these circRNAs indicated five potential pathways that are related to AF progression, including dilated cardiomyopathy, hypertrophic cardiomyopathy, signaling pathways regulating pluripotency of stem cells, the Hippo signaling pathway, and the TGF-β pathway. Similar circRNA-miRNA interaction trends were reported in CHD patients by Lin et al.[76] 110 circRNAs were differentially expressed in CHD patients and healthy individuals. Enrichment analysis showed that the mechanisms of circRNA-modulated CHD development include multiple pathways and cellular and biological processes. Kunzhe et al.[77] identified many circRNAs that were produced from known cardiac disease-related loci and were dysregulated in dilated cardiomyopathy (DCM). In addition, a novel class of circRNA, fusion-circRNAs, was reported. These fusion-circRNAs are highly conserved among different species and have strong potential to sponge DCM-related miRNAs. With the databank of circRNA ceaselessly expanding, it is possible to establish a database of circRNA biomarkers, or a network of circRNAs, yet it is far from completion. Such databases could provide a new diagnostic model of CVDs and even lead to a whole new aspect of pharmaceutical targeting in these diseases. Future research should not only focus on identifying circRNAs in order to enrich our databank, but also investigate the underlying upstream and downstream biological pathways to further understand circRNA-related pathogenesis.

Proof-of-Principle Study of circRNA in CVD

To date, only a few studies have investigated the functions of circRNA in disease pathogenesis. Several studies attempted to attenuate diseases by manipulating circRNA expressions (Table 2). The circular transcript of the sodium/calcium exchanger 1 (NCX1) gene is one of the most heavily focused circRNAs linked to cardiomyopathy. Li et al.[78] investigated the role of circNCX1 (formal name circSlc8a1) in oxidative stress-induced cardiomyocyte apoptosis during ischemic myocardial injury. The interaction of circNCX1 with miRNA was examined by AGO2-IP and RNA pull-down assays by using a myocardial I/R mouse model. circNCX1 promotes cardiomyocyte apoptosis by acting as an endogenous miR-133a-3p sponge. CDIP-1, a pro-apoptotic gene, was decreased by knockdown of circNCX1 in cardiomyocytes and heart tissues, and hence reduced the apoptosis and I/R injury. Lim et al.[79] also investigated the role of circSlc8a1 in cardiomyocytes. *In vivo*, AAV9-mediated RNAi knockdown of cricSlc8a1 leads to attenuation of heart hypertrophy while overexpression of circSlc8a1 resulted in HF. Since miR-113a was highly enriched in the fraction of circSlc8a1 pull-down, molecular analysis of miR-113a showed that its targets serum response factor (Srf), connective tissue growth factor (Ctgf), adrenoceptor beta 1 (Adrb1), and adenylate cyclase 6 (Adcy6) can be regulated by circSlc8a1, indicating that circSlc8a1 may serve as miRNA sponges for miR-113a in cardiomyocytes and therefore may be a novel therapeutic target for cardiac hypertrophy.[79] The same circRNA circSlc8a1 was also found to be downregulated in bladder cancer, and its expression led to inhibition of cancer cell invasion and migration. By sponging miR-130b and miR-494, the tumor suppressor gene PTEN pathway was activated.[80] Different studies have demonstrated that circSlc8a1 is involved in multiple pathogenic pathways, and thus it is reasonable to predict that circSlc8a1 could be involved in other unknown pathways. Further clarification is needed.

Human circFndc3b is significantly downregulated in ischemic cardiomyopathy patients. Overexpression of circrFndc3b by AAV9 reduced cardiomyocyte apoptosis, enhanced neovascularization, and further improved heart function.[81] Another study focused on the circRNA circHRCR acting as an endogenous miRNA-223 sponge to inhibit cardiac hypertrophy and HF.[82] They found HRCR functions with endogenous miR-223 activity, which resulted in the increase of the miR-223 downstream target (ARC). The expression of HRCR in cardiomyocytes and in mice both showed attenuation of hypertrophic responses. It seems that many circRNAs act as miRNA sponges in cardiopathy such as HF (HRCR binding miRNA-223),[82] MI (circRNA-MICRA act as a sponge of miR-150; circRNA_081881 contains seven binding sites for miR-548),[62,83] I/R injury (circ-NCX1 acts as an endogenous miR-133a-3p sponge),[78] and cardiac fibrosis (circRNA_000203 acts as an miR-26b-5p sponge).[84] In addition, there are also some circRNAs that play key roles in cardiomyopathy and CVD by other pathways. circ-Amotl1 was found to interact with PDK1 and AKT1, facilitating the nuclear translocation of AKP and PDK1, reducing apoptosis, hypertrophy, and fibrosis.[85] circFoxo3 was found to relate to cardiac senescence by binding ID1, E2F1, HIF1a, and FAK and decreasing their translocation into the nucleus.[86] circANRIL controls pre-rRNA maturation and nucleolar stress through interaction with multiple RBPs, acting as a protective factor against atherosclerosis.[87]

Zhu et al.[88] reported decreased expression of circRNA circNFIB in mouse post-MI samples. Inhibition of circNFIB promotes fibroblast proliferation while overexpression of the circRNA attenuates pro-proliferative effects, indicating the potential of a novel therapeutic approach for treatments of fibrotic diseases. Li et al.[89] used a rat embryonic ventricular cardiomyocyte-derived H9c2 cell line to study circRNA interaction with BCL2 interaction protein 3 (BNIP3), which

Table 2. Recent Top-Studied circRNAs in Pathogenic Hearts

Disease	circRNA Being Investigated	Parental Gene	Mechanism	Downstream Effect by Knockout	Downstream Effect by Expression	Ref.
Ischemic myocardial injury	circNCX1(circSlc8a1)	sodium/calcium exchanger 1 (NCX1)	miR-113a-3p sponge			78,79
	circFndc3b	fibronectin type III domain-containing protein 3B	FUS/VEGF-A axis		reduced cardiomyocyte apoptosis, enhanced neovascularization	81
Hypertrophy heart failure	HRCR		miR-223 sponge		ARC pathway and reduced hypertrophy	82
Myocardial infarction	circRNA-MICRA		miR-150 sponge			62
	circRNA_081881		miR-548 sponge			83
Cardiac fibrosis	circRNA_000203		miR-26b-5p sponge			84
Hypertrophy	circ-Amotl1		interacts with PDK1 and AKT1		nuclear translocation of AKP and AKT1, decreased apoptosis	85
Hypertrophy (mouse)	circSlc8a1	sodium/calcium exchanger 1 (NCX1)	miR-133a sponge	increased heart function, decreased left ventricular septal thickness, decreased cardiomyocyte width, decreased heart weight	increased heart weight	79
Cardiac senescence	circFoxo3		binds ID1, E2F1, HIF1a, FAK		decreased ID1, E2F1, HIF1a FAK nuclear translocation	86
Atherosclerosis	circANRIL		controls pre-rRNA maturation		protective factor	87
Myocardial infarction (mice)	circRNA-circNFIB		miR-433	attenuated fibroblast pro-proliferation	promoted fibroblast proliferation	88
Hypoxia-induced cardiac myocyte apoptosis, H9c2 cell	hsa_circ_0005972	BNIP3	miR-27a-3p sponge	reversed apoptosis		89
	hsa_circ_0020526					
	hsa_circ_0020527					
Cardiac fibrosis	mmu_circ_0001052	HIPK3	miR-29b-3p sponge	suppressed proliferation of cardiac fibroblasts, decreased fibrosis area	increased cardiac fibroblast proliferation	90

is upregulated in hypoxia and participates in hypoxia-activated apoptosis of cardiac myocytes. Three circRNAs transcribed from BNIP3 gene were identified, including hsa_circ_0005972, hsa_circ_0020526, and hsa_circ_0020527. These circRNAs were found to be upregulated in hypoxia-induced injury of H9c2 cells. The BNIP3 circRNAs were then knocked down and cell viability was evaluated by Cell Counting Kit-8 (CCK8) and 5-ethynyl-2′-deoxyuridine (EdU) assays. Results showed a reversed injury of H9c2 cells. This group further investigated the mechanism of circRNA interacting with BNIP3, and they found that those circRNAs sponged miR-27a-3p to upregulate BNIP3 expression in H9c2 cells. Ni et al.[90] focused on HIPK3 gene-derived circHIPK3 (mmu_circ_0001052). They discovered that circRNA expression levels were high in cytoplasm in most tissues except in liver. With a higher half-life and resistance to RNase R, circHIPK3 showed a significant increase at 24 h after treatment with both TGF-β and angiotensin II (Ang II), since they both triggered cardiac fibrosis. Transfection of siRNA targeting circHIPK3 significantly reduced circHIPK3 levels while mRNA expression remains unchanged. The transfected cells showed suppressed proliferation of CF induced by Ang II, while no difference could be observed in cells transfected with HIPK3 siRNA, indicating that CF proliferation relied on circHIPK3 but not HIPK3. To understand the underlying mechanism of circHIPK3 in CF progression, immunoprecipitation and bioinformatics analyses were performed. Among several miRNA candidates, miR-29b-3p was found colocalizing with circHIPK3 to the cytoplasm in CFs, indicating that circHIPK3 probably served as a miRNA sponge in CFs. *In vivo* study showed that silencing circHIPK3 or miR-29-3p overexpression significantly decreased fibrosis area (%) in an Ang II-induced cardiac fibrosis mouse model, therefore concluding that circHIPK3 and miR-29-3p were involved in Ang II-induced cardiac fibrosis, probably by circRNA sponges.

Conclusions and Perspectives

circRNAs are no longer overlooked as by-products of translation: studies are conducted with increasing magnitude and expansion to different fields. The discovery and analysis of circRNAs require both bench work and computational technology. Evidence has already proven that circRNAs may be promising biomarkers for major diseases such as CVD and cancer. However, what we have known about circRNAs is only the tip of the iceberg. There are more than thousands of types of circRNAs that are yet to be fully investigated.

Furthermore, the experimental models and methods of research still require optimization. For CVD specifically, the complexity of heart tissue and its crucial functions make experiments not as easily accessible. Tremendous amounts of data should be collected before clinical implementation. Upon completion of a database and application of artificial intelligence technology, early diagnosis is possible if the patient's non-coding RNA data could be compared and analyzed. Exosomal circRNAs should be a starting point of circRNA application since it is easily accessed through serum collection. Such non-invasive test data could be a reference for diagnosis, prognosis, and monitoring in a variety of diseases in the future. With the novel method of diagnosis or treatment of long-term CVD at an early stage, or a predictor before the onset of clinical symptoms, there may be a significant improvement in healthcare. Earlier diagnosis can potentially increase life expectancy and lower personal and governmental healthcare costs in the long run.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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