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Circular RNAs are a novel type of non-coding RNAs in ROS regulation, cardiovascular metabolic inflammations and cancers

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Abstract

Circular RNAs (circRNAs) are a novel class of endogenous non-coding RNAs characterized by a covalently closed-loop structure generated through a special type of alternative splicing termed back-splicing. Currently, an increasing body of evidence has demonstrated that *1)* majority of circRNAs are evolutionarily conserved across species, stable, and resistant to RNase R degradation, and often exhibit cell-specific, and tissue-specific/developmental-stage-specific expression and can be largely independent of the expression levels of the linear host gene-encoded linear RNAs; *2)* the biogenesis of circRNAs via back-splicing is different from the canonical splicing of linear RNAs; *3)* circRNA biogenesis is regulated by specific cis-acting elements and trans-acting factors; *4)* circRNAs regulate biological and pathological processes by sponging miRNAs, binding to RNA-binding protein (RBP), regulators of splicing and transcription, modifiers of parental gene expression, and regulators of protein translation or being translated into peptides in various diseases; *5)* circRNAs have been identified for their enrichment and stability in exosomes and detected in body fluids such as human blood, saliva, and cerebrospinal fluids, suggesting that these exo-circRNAs have potential applications as disease biomarkers and novel therapeutic targets; *6)* several circRNAs are regulated by oxidative stress and mediate reactive oxygen species (ROS) production as well as promote ROS-induced cellular death, cell apoptosis, and inflammation; *7)* circRNAs have also emerged as important regulators in atherosclerotic cardiovascular disease, metabolic disease, and cancers; *8)* the potential mechanisms of several circRNAs have been described in diseases, hinting at their potential applications as novel therapeutic targets. In this highlight, we summarized the current understandings of the biogenesis and functions of circRNAs and their roles in ROS regulation and vascular inflammation-associated with cardiovascular and metabolic disease.

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Declaration of Competing Interest

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Keywords

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1. Introduction

Cellular ribonucleic acids (RNAs) can be divided into two categories: coding and non-coding RNAs. Non-coding RNAs (ncRNAs) are functional RNAs that are transcribed from DNA, but are incapable of being translated into proteins. These ncRNAs can be classified into two groups based on their sizes, ncRNAs greater than 200 nucleotides are classified as long non-coding RNAs (lncRNAs), and ncRNAs less than 200 nucleotides are grouped as small ncRNAs. Small ncRNAs can be further classified into: microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) (Esteller, 2011; Hombach & Kretz, 2016; Mattick & Makunin, 2006) (Fig. 1). In recent years, a large number of studies have shown that ncRNAs play fundamental regulatory roles in biological processes (Wei, Huang, Yang, & Kang, 2017). One of the significant examples in demonstrating the master gene functions of these ncRNAs is that we established the first mouse model of metabolically healthy obesity with miR155 deficiency in atherogenic apolipoprotein E deficient ($ApoE^{-/-}$) background (Johnson et al., 2018; Virtue et al., 2017; Virtue, Wang, & Yang, 2012).

In addition to lncRNAs and small ncRNAs, circular RNAs (circRNAs) represent a novel and large family of non-coding endogenous RNAs recently discovered in all eukaryotic cells. These ncRNAs arise from a particular alternative splicing (back-splicing) mechanism of precursor mRNAs (pre-mRNAs) (Chen, Huang, Wang, & Shan, 2015a). This back-splicing mechanism results in a covalently closed circular loop molecule that lack $5' - 3'$ ends and poly-adenylated tails (Salzman, 2016). Compared to the other types of ncRNAs, circRNAs are evolutionarily conserved among species, highly stable, and resistant to RNase R degradation (Table 1). These features provide circRNAs with many potential functions, such as (a) acting as miRNA sponges, (b) regulating the expression of parental genes, (c) regulating alternative splicing or translation, (d) acting as RNA-binding protein (RBP) sponges, and (e) being translated into peptides/proteins.

CircRNAs were discovered more than four decades ago and were first identified in plant-based viruses via electron microscope in 1976 (Sanger, Klotz, Riesner, Gross, & Kleinschmidt, 1976). In 1979, circRNAs were identified in eukaryotic cells as endogenous RNA splicing products (Hsu & Coca-Prados, 1979). Then in 1986, circRNAs were identified in humans following hepatitis delta virus infection (Kos, Dijkema, Arnberg, van der Meide, & Schellekens, 1986). Therefore, circRNAs were typically considered as a functionless byproducts of aberrant RNA splicing and thus have not gained sufficient scientific attention. Two reports significantly transformed the field: the first study showed that RNA transcripts from many human genes were arranged in a non-canonical order, resulting in a type of circRNAs isoform (Salzman, Gawad, Wang, Lacayo, & Brown, 2012). The second study reported that circular transcripts of cerebellar degeneration-related protein 1 antisense RNA

(CDR1as, ciRS7) could act as miRNA sponges for miR-7 (Memczak et al., 2013). These significant findings changed circRNAs into a new focal point of scientific research and rising stars in the ncRNA field. An excellent RNA database, exoRBase (http://www.exorbase.org/), has collected as many as 58,330 circRNAs, 15,501 lncRNAs, and 18,333mRNAs (Li et al., 2018a), suggesting that the numbers of circRNAs are even higher than that of mRNAs.

In recent years, the development and application of microarray techniques, high-throughput deep RNA sequencing (RNA-seq), and novel bioinformatics approaches have led to the discovery of many circRNAs (Broadbent et al., 2015; Danan, Schwartz, Edelheit, & Sorek, 2012; Fan et al., 2015; Lu et al., 2015; Yang, Duff, Graveley, Carmichael, & Chen, 2011) with some studies suggesting they may outnumber mRNAs (Li, Li, et al., 2018a). These recent advancements have demonstrated that circRNAs are incredibly abundant, relatively stable, diverse and conserved, and broadly expressed in eukaryotic cells (Jeck et al., 2013). Additionally, circRNAs exhibit cell-type, tissue-type or developmental stage-specific expression (Conn et al., 2015; Guo, Agarwal, Guo, & Bartel, 2014; Salzman et al., 2012; Salzman, Chen, Olsen, Wang, & Brown, 2013). Emerging evidence suggests that circRNAs are responsible for regulating complicated biological functions. For example, studies have shown a role for circRNAs as endogenous microRNA (miRNA) sponges (Hansen et al., 2013; Memczak et al., 2013; Wang et al., 2016a), transcriptional regulators of their encoding parental gene expressions (Li et al., 2017a; Zhang et al., 2013), and modulating alternative splicing (Ebbesen, Hansen, & Kjems, 2017). Furthermore, circRNAs can interact with RBPs, and act as scaffolds in the assembly of protein complexes (Du et al., 2016; Zhang et al., 2017; Zhang & Xin, 2018).

Recent studies have implicated circRNAs in physiological processes such as aging (Westholm et al., 2014) and insulin secretion (Xu, Guo, Li, & Yu, 2015). Additionally, circRNAs have been demonstrated to play a critical role in the pathogenesis of various human diseases, including atherosclerotic cardiovascular disorders (Burd et al., 2010; Holdt et al., 2016), diabetes (Zhao et al., 2017a), cancers (Bachmayr-Heyda et al., 2015; Chen et al., 2017a; Geng, Jiang, & Wu, 2018; Qin et al., 2016), Alzheimer's disease (Lukiw, 2013; Zhao, Alexandrov, Jaber, & Lukiw, 2016), nervous system disorders (Bai et al., 2018), and osteoarthritis (Liu et al., 2016). In particular, we (Li et al., 2019a) and others reported that circRNAs are associated with cardiovascular and metabolic diseases; however, little is known about the exact role of circRNAs in vascular inflammation associated with cardiovascular and metabolic diseases.

Several studies reported that circRNAs can perform their biological functions inside the cell or exported by exosomes and taken up by neighboring (paracrine) or distant cells (endocrine) and affect many aspects of physiological and pathological conditions of the recipient cells (Bai, Lei, Huang, Jiang, & Zhou, 2019; Li et al., 2015a). Paracrine signaling has been proposed to promote cell-cell communication in various human cancers (Dou et al., 2016; Hon, Ab-Mutalib, Abdullah, Jamal, & Abu, 2019; Li, Zheng, et al., 2015a; Louis, Desoteux, & Coulouarn, 2019). However, the roles of exosomal circRNAs in propagating cardiovascular and metabolic inflammation have not been studied. Furthermore, the

development of novel therapeutics targeting circRNAs may help mitigate vascular inflammation associated with cardiovascular and metabolic diseases.

2. CircRNA biogenesis

CircRNA biogenesis adds a modification step to the conventional generation of mRNAs. The precursor mRNA (pre-mRNA) splicing is catalyzed by the canonical spliceosome machinery to remove introns from the transcript and join exons leading to the formation of a linear mRNA with 5′–3′ polarity. However, circRNAs are generated from pre-mRNA by the action of RNA polymerase II (Chen, 2016). Most circRNAs do not follow the canonical 5′–3′ order, and are produced during back-splicing (Vicens & Westhof, 2014). These circRNAs are distinct from their linear RNA counterparts because they lack the 5′ – 3′ ends and poly-adenylated tail due to their closed covalent structure, which usually decides the fate of many RNA transcripts (Memczak et al., 2013). In general, circRNAs originated from exonic sequences, producing exonic circRNAs (ecircRNAs) (Jeck et al., 2013); or from intronic sequences, producing intronic circRNAs (ciRNAs) (Zhang et al., 2013). However, the exon-intron circRNAs (EIciRNAs) can be generated from intron-containing exons (Jeck & Sharpless, 2014). Exonic circRNAs are formed as a result of pre-mRNA splicing where the 3′ splice donor attaches to the 5′ splice acceptor forming an exonic circRNA (Jeck et al., 2013; Jeck & Sharpless, 2014). This type of splicing might occur with a single exon, but sometimes happens with multiple exons. However, if the introns between the exons are retained, the resulting circRNAs are referred to as exon-intron circRNAs (Li et al., 2015b). The intronic circRNAs are produced from intron lariat (Jeck et al., 2013; Li, Huang, et al., 2015b). The process of intronic circRNAs formation depends on GU-rich sequences near the 5’ splice site and C-rich sequences near the branch point. Finally, the lariat undergoes debranching and easily degraded while the mature intronic circRNA is generated (Barrett, Parker, Horn, Mata, & Salzman, 2017). These circRNAs are formed in the nucleus, and some of them are transported to the cytoplasm. The exonic circRNAs typically reside in the cytoplasm, but the intronic and exon intronic circRNAs remain in the nuclei (Li, Huang, et al., 2015b) (Fig. 2A).

To date, four different hypothetical models of circRNA biogenesis have been proposed (Fig. 2B) including (i) intron-pairing-driven circularization; (ii) RNA binding protein (RBP)-driven circularization; (iii) exon-skipping; and (iv) intron lariat circularization (Barrett, Wang, & Salzman, 2015). The first model of circRNA biogenesis is intron pairing-driven circularization (Fig. 2Bi). In this model, two introns flanking the exon/exons of a pre-mRNAhave a structure capable of joining each other. The flanking introns approach each other creating a secondary conformation that makes the splice sites possible to carry on back-splicing and generate exonic circRNA. Adenosine deaminase 1 acting on RNA (ADAR1) is involved in the intron-pairing process of circRNA formation (Athanasiadis, Rich, & Maas, 2004). The second proposed model is the RBPs-driven circularization (Fig. 2Bii). In this model, RBPs bind to pre-mRNAs to connect the flanking introns. This process is induced by protein dimerization, which forms an RNA loop. Muscleblind like splicing regulator 1 (MBNL1) protein, is the most popular RBPs responsible for circRNA biogenesis (Chen & Yang, 2015). The third circRNA biogenesis model that can give rise to back-splicing is exon skipping (Fig. 2Biii). In this model, one or multiple exons of the mature

mRNA will be missing. The lariat-driven circularization proceeds as the non-adjacent exons join producing linear mRNA and exon-intron or multiple exon circRNA transcript with lariat structure. Finally, the fourth proposed mechanism is the intron lariats (Fig. 2Biv) which can form intronic circRNAs (ciRNAs) (Kristensen et al., 2019). These three major subclasses of generated circRNAs are different in their location, formation, and biological function (Fig. 3).

3. Regulation of circRNA biogenesis

In general, the levels of steady-state circRNA expression in cells can be regulated by transcription regulation of circRNA-producing pre-mRNA or by circRNAs degradation. Emerging lines of evidence show that the transcriptional regulation of circRNAs involves both cis-regulatory elements and trans-acting factors that control the back-splicing machinery required for circRNA biogenesis. These factors include core spliceosome components, intronic complementary sequences (ICSs) flanking circle formation exons, and other regulatory RBPs. Both cis-elements and trans-factors are required to bring the downstream donor and upstream acceptor sites close together to promote back-splicing (Starke et al., 2015; Zhang et al., 2014a).

Previous studies reported that both canonical splice signals and spliceosome machinery are required for back-spliced circularization; and that back-splicing is usually coupled with canonical splicing (Ashwal-Fluss et al., 2014; Starke et al., 2015). It has been reported that the processing of circRNAs can be facilitated by either RNA pairing of reversely complementary sequences across their flanking introns or protein factors binding to pre-mRNAs to bridge flanking introns together (Ashwal-Fluss et al., 2014; Starke et al., 2015; Zhang, Wang, et al., 2014a). RNA pairing across flanking introns promotes exon circularization; and strong pairing capacity could dramatically increase the production of circRNAs. Two known RNA binding proteins, MBNL1 and ADAR1, are reported to play a significant role in circRNA biogenesis, where they bind to its own pre-mRNA and bridge two flanking introns close together leading to increased circRNA formation (Ashwal-Fluss et al., 2014; Ivanov et al., 2015). Notably, ADAR1 knockdown increased expression levels of some circRNAs, indicating a role of ADAR1 in the suppression of circRNA biogenesis (Chen et al., 2015b).

4. Properties of circRNAs

CircRNAs have several important properties including: *1)* circRNAs are exist as endogenous ncRNA (Salzman et al., 2012); *2)* circRNAs are widely expressed in eukaryotic cells (Jeck et al., 2013), and exhibit cell-specific and tissue-specific/developmental-stage-specific expression (Memczak et al., 2013; Xu,Wu, Han, Zhao, & Song, 2017). Despite the low levels of most circRNA expression, some are highly expressed than their corresponding linear mRNAs (Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2012). Previous studies reported that the expression of some circRNAs is more than 10-fold higher than the linear mRNA counterparts (Jeck et al., 2013). Also, other studies demonstrated that the expression of circRNA is not correlated with the expression of its linear mRNA as some circRNA is not detected even though the mRNA expression levels is too high (Nigro et al.,

1991). Others demonstrated that circRNAs are highly expressed in the brain tissue (Rybak-Wolf et al., 2015). Different biological processes including epithelial-mesenchymal transition (EMT) (Conn et al., 2015), aging, and stress significantly changes the expression profile of circRNAs (Cortés-López et al., 2018; Fischer & Leung, 2017). Furthermore, RNA-seq analysis of human fetal and adult tissues including liver, kidney, heart, lung, stomach, and colon reported that up to 50% of circRNAs are tissue specific and their expression level is higher in fetal tissues than in adult tissues (Xu et al., 2017). Additionally, circRNAs are expressed at very low levels in human cancer cells (Bachmayr-Heyda et al., 2015). These studies strongly suggest that the expression levels of circRNA is a highly regulated process; *3)* compared to linear RNAs, circRNAs form covalently closed-loop structures with neither $5'-3'$ polarities nor poly-adenylated tails, resulting in reduced degradation by RNA exonuclease or RNase R, which makes them more stable than linear RNAs (Suzuki et al., 2006; Suzuki & Tsukahara, 2014); *4)* The majority of the circRNAs including exonic circRNA are predominantly found in the cytoplasm (Jeck et al., 2013; Jeck & Sharpless, 2014; Memczak et al., 2013), however, few of them including the intron retained circRNAs such as intronic circRNA and exon-intron circRNA are exclusively located in the nucleus (Ebbesen et al., 2017; Zhang et al., 2013; Zhang, Wang, et al., 2014a). It was reported previously that some exonic circRNAs are also found in the nucleus (Errichelli et al., 2017) then exported to the cytoplasm. However, the mechanism by which circRNAs are exported from the nucleus to the cytoplasm remains unknown. As in the aforementioned, there are several models of circRNA biogenesis were hypothesized including lariat-driven circularization or exon skipping that generate intronic or exonic-intronic circRNAs and intron-pairing-driven circularization or direct back-splicing that generate exonic circRNA. Of note, previous studies demonstrated that intron-pairing driven circularization may occur more frequently than lariat-driven circularization (Jeck & Sharpless, 2014). Therefore, these studies indicates that exonic circRNAs represent the largest class (more than 80%) of total circRNAs and locate in the cytoplasm, while intronic circRNA represents only 20%(Chen, Huang, et al., 2015a) (Fig. 3).; *5)* most of circRNAs are located in the cytoplasm and few are found in the nucleus (Memczak et al., 2013; Zhang et al., 2013); *6)* circRNAs are highly conserved between species (Jeck et al., 2013; Salzman et al., 2012; Zhang, Wang, et al., 2014a); *7)* most of circRNAs have microRNA response element (MRE), so they can interact with miRNAs and thus regulate target gene expressions (Hansen et al., 2013); and *8)* vast majority of circRNAs can play roles in transcription and post-transcription regulation (Zhang et al., 2013).

5. Biological functions and mechanisms of circRNAs

In recent years, the biological functions of circRNAs have become a hotspot of scientific research. A growing body of evidence shows that besides acting as miRNA sponges (Bartel, 2009), several other roles of circRNAs have been proposed (Fig. 4). CircRNAs may also interact with regulatory RBPs through their activities as protein sponges, decoys, scaffolds, and recruiters (Huang, Zheng, Wu, Chen, & Huang, 2020). By enhancing binding to RNA polymerase II, circRNAs located in the nucleus may modulate the transcription of their host genes, and regulate alternative splicing and transcription as well as translation (Conn et al., 2017).

5.1. CircRNAs can act as miRNA sponges

The majority of circRNAs are located in the cytoplasm, indicating that the circRNA is involved in post-transcriptional regulation and might function as miRNA sponges or competitive endogenous RNAs (ceRNAs) to regulate the expression of miRNA targets (Jeck et al., 2013). MicroRNAs can bind to the complementary sequences in the 3′-untranslated regions of mRNAs (Bartel, 2004) to facilitate mRNA degradation. Many circRNAs contain miRNA binding sites, also known as miRNA response elements (MREs), which allow them to sponge miRNAs and act as ceRNAs in order to inhibit miRNAs from negatively regulating their target mRNAs (Memczak et al., 2013). By binding to the 3'-untranslated region (UTR) of mRNAs, miRNAs can inhibit protein translation and facilitate mRNA degradation (Bartel, 2004) (Fig. 4A).

Since miRNAs have an inhibitory effect on their target genes, circRNA sponging leads to the upregulation of the miRNA target mRNAs and increases the expression of that gene products. Therefore, circRNAs indirectly regulate mRNAs translation. However, it is important to consider the stoichiometric relationship between the miRNA binding sites of the circRNA and the mRNA target sites of the miRNA. Of note, highly abundant circRNAs having many competing binding sites are more likely to have competing endogenous RNA function (Thomson & Dinger, 2016). For example, CDR1as, a circRNA that is highly expressed in the brain tissue, has more than 70 conserved seed-binding sites for miR-7, and miR-7 occupies many of these sites (Piwecka et al., 2017), which is involved in the regulation of several miR-7 target genes in the brain (Hansen et al., 2013; Memczak et al., 2013). Similarly, miR-671 has one complementary binding site to CDR1as, indicating that while miR-671 can mediate cleavage of CDR1as, CDR1as might regulate miR-7 levels and activity (Hansen et al., 2011; Hansen et al., 2013). Furthermore, the circRNA Sry acts as a miRNA sponge for miR-138, with at least 16 binding sites for miR-138 (Hansen et al., 2013). On the other hand, some circRNAs do not possess multiple binding sites for a specific miRNA, but they harbor many different types of miRNA binding sites. For instance, circRNA circITCH has multiple binding sites for miR-7, miR-17, and miR-214 leads an increase of E3-ubiquitinligase gene (INCH) expression (Li et al., 2015c). Even though, some identified circRNAs possess fewer miRNA binding sites than their co-linear mRNAs counterpart, but also exhibit their miRNA sponge function, indicating that the function of circRNAs as miRNA sponges is conserved across species. Recently, many studies have determined the sponge function of circRNAs; for example, circ_CHFR has been shown to promote oxidized low-density lipoprotein (oxLDL)-induced vascular smooth muscle cell proliferation, migration, and inflammation by interacting with miR-214–3p (Zhuang et al., 2020). In addition, homeodomain-interacting protein kinase 3 (HIPK3) circular RNA (circHIPK3) suppresses miR-30a-3p activity by sponging miR-30a-3p (Shan et al., 2017). These studies indicated that circRNAs might play a significant role as miRNA sponges in inflammatory cardiovascular diseases.

5.2. CircRNAs can act as RNA-binding protein (RBP) sponges

RBPs play a critical role in post-transcriptional regulatory processes associated with different biological activities, including cell proliferation, apoptosis, senescence, and cell responses to oxidative stress via post-transcriptional regulation (Brinegar & Cooper, 1647).

Increasing evidence suggests that specific circRNAs can act as protein sponges by providing binding sites for specific RBPs to sequester and inhibit the biological activity of proteins through competitive inhibition (Zang, Lu, & Xu, 2020) (Fig. 4B)). The interaction of circRNAs with the RBPs such as Elav like RNA binding protein 1 (HuR), KH-type splicing regulatory protein (KHSBP), tristetraprolin (TTP), heterogeneous nuclear ribonucleoprotein D, F-box family protein (AUF1), and other potent regulatory RBPs could indirectly affect the fate of their respective target mRNAs (Panda, Grammatikakis, Munk, Gorospe, & Abdelmohsen, 2017a). We and our collaborators recently reported that in a bone marrow (BM) transplantation mouse model with TTP deficient BM transplantation into low-density lipoprotein receptor deficient recipient mice ($LDLR^{-/-}$), TTP deficient ($TTP^{-/-}$) BM recipients display significantly higher systemic and multi-organ inflammation than $TTP^{+/+}$ BM recipients, implying that the TTP-sponged circRNAs could enhance systemic inflammation (Saaoud et al., 2020). Previous studies showed that circFoxo3, the circular RNA variant of the Foxo3 gene, can interact with p21 and cyclin dependent kinase 2 (CDK2) to inhibit cell cycle progression. CircFoxo3 can also bind and inhibit senescence-associated proteins (ID1 and E2F transcription factor 1, E2F1) and stress-related proteins (hypoxia-inducible factor 1, HIF1a and focal adhesion kinase, FAK) in the cytoplasm to regulate cardiac senescence (Du et al., 2016; Du et al., 2017).

5.3. CircRNAs can regulate translation and alternative splicing

Besides acting as miRNA sponges and binding to RBPs, circRNAs have a potential role in regulating protein translation (Jeck & Sharpless, 2014) (Fig. 4C). Several circRNAs can bind open reading frames (ORFs) and be translated as protein fragments. It has been shown that protein/polypeptides can be coded by circRNAs both *in vivo* and *in vitro* only when the RNAs contain prokaryotic ribosome-binding sites, or internal ribosome entry site elements (IRES) (Chen & Sarnow, 1995; Perriman & Ares Jr., 1998). It has been confirmed that circ-ZNF609 can be translated into a protein functioning in myogenesis (Legnini et al., 2017). Recently, it has been reported that N^6-methyladenosine (m^6A) is the most common and abundant base methylation modification of RNAs and promotes the initiation of protein translation from circRNA in human cells. Many circRNAs contain the m^6A motifs, and only one m^6A is required to drive translation initiation (Yang et al., 2017a). Interestingly, a large number of circRNAs have the potential for translation, indicating that they may have a regulatory role in the stimulation of circRNA-derived proteins in cell responses to environmental stressors. Besides being translated themselves, there is emerging evidence that some circRNAs, including circPABPN1, can regulate translation of their associated linear mRNAs (Abdelmohsen et al., 2017). These studies provide a new direction for the functional studies of circRNAs.

Previous studies reported that ecircRNAs could play a role in alternative splicing as the circularization can compete with canonical splicing. Mannose-binding lectin 2 (MBL2) protein is a splicing factor, which can affect alternative MBL pre-mRNA splicing during the generation of MBL mRNA and circular MBL (circMBL). The second exon of the MBL gene contains sequences that form a circRNA transcript having conserved binding sites for MBL protein in the flanking intronic sequences. In an auto-regulatory manner, circMBL influences the selective splicing of the MBL mRNA. Additionally, MBL can interact with

circMBL and its flanking introns to promote exon circularization. Therefore, the competition between back-splicing for circRNA generation and canonical splicing is evident from the concomitant reduction in circRNA and an increase in linear splicing (Ashwal-Fluss et al., 2014). These findings may hint that some circRNAs may play a role in controlling the expression of mRNA through binding to RBPs and affecting the canonical splicing.

5.4. CircRNAs act as gene expression regulators

There are competitions between the biosynthesis of circRNAs and linear RNAs, with the expression of linear RNAs being regulated by circRNAs. Recent studies showed that certain circRNAs such as intron (ciRNAs) and intron-exon (EIciRNAs) circRNAs can promote transcription of the parental genes. These circRNAs have few binding sites for miRNAs, suggesting that they may function differently (Zhang, Yang, & Chen, 2014b). Also, ciRNAs and EIciRNAs are widely localized and detected in the nuclei and act as transcriptional regulators by binding to U1 small nuclear ribonucleoproteins (U1 snRPN) and RNA polymerase II in the promotor region of genes, thus promoting the transcription of the parental gene (Ashwal-Fluss et al., 2014; Li, Huang, et al., 2015b) (Fig. 4D).

6. Degradation and elimination of circRNAs

Once generated, exonic circular RNAs progressively accumulate in the cytoplasm. CircRNAs are highly stable and have long half-lives because of their circular structure, which makes them naturally resistant to degradation by RNase exonucleases. Currently, very little is known about the mechanisms of how circRNAs are degraded *in vivo*. The first evidence of natural circRNAs degradation via endonuclease activity was found *in vitro* using RNase H and DIS3 homolog, exosome endoribonuclease and 3’−5’ exoribonuclease (Rrp44) (Zhao, Zhu, Limbo, & Russell, 2018). Also, RNase L has been identified globally to degrade circRNAs (Liu et al., 2019a) (Fig. 4E). Additionally, circRNA binding by miRNAs may initiate circRNA decay by Argonaute RNA-induced silencing complex (RISC) catalytic component 2 (Ago2)-mediated cleavage. For instance, circRNA CDR1as degradation was dependent on miR-671-mediated Ago2 cleavage (Hansen et al., 2011). Emerging studies have demonstrated that circRNAs are abundant and stable in exosomes. These exosomal circRNAs were identified and detected in human blood and urine (Li, Zheng, et al., 2015a; Memczak, Papavasileiou, Peters, & Rajewsky, 2015) (Fig. 4F). Furthermore, multiple circRNAs are secreted from cells into extracellular space by exosomes and further removed by the reticulo-endothelial system or eliminated by the kidney and liver (Choi & Lee, 2016; Lasda & Parker, 2016; Wang et al., 2019a). Recently, the RNA modification, N^6-methyladenosines (m^6A), as well as poly (I:C) stimulation were shown to mediate the activation of the endoribonuclease, RNase L, and ultimately the degradation of both mRNAs and circRNAs (Park et al., 2019).

7. Approaches studying circRNAs

Since the initial discovery of circRNAs, various biochemical tools have been discovered and identified to detect and validate the existence of circRNAs and their localization, biogenesis, biological functions, disease implication, interacting molecules, and therapeutic potential. Furthermore, online resources, bioinformatics and statistical approaches have been

developed to quantify the expression of circRNAs and identify new circRNAs with high confidence (Li, Yang, & Chen, 2018b) (Table 2A). First, *high-throughput circRNA sequencing (circ-seq) analysis* has enabled the identification of thousands of circRNAs. The unique feature of this technique is that circRNAs are enriched with RNase R digestion to eliminate most linear transcripts and keep circRNAs intact (Jeck et al., 2013; Salzman et al., 2013). Second, *microarray high-throughput analysis* is another useful technique to detect the expression levels of specific circRNAs. This method typically requires pre-treatment of the RNA samples with RNase R to degrade linear transcripts and enhance circRNA detection. The disadvantages of this technique include: 1) microarray platforms incorporate only a limited number of known circRNAs, 2) circRNAs identification is based only on the junction sequences and circRNAswith shared junction sequences cannot be distinguished, and 3)microarray cannot inform on the internal sequence of a given circRNA (Li et al., 2016a; Qu et al., 2015). A third technology used to study circRNA is *reverse transcription-polymerase chain reaction (RT-PCR)* has been used to validate the high-throughput circ-seq and microarray analysis. This technique requires the use of divergent primers spanning the circRNA junction, and the extracted circRNAs must be enriched with RNase R followed by RT-qPCR analysis, but often this step can be avoided if the circRNA is abundant, as divergent primers do not amplify linear RNA (Panda, Abdelmohsen, & Gorospe, 2017b). Fourth, *digital droplet PCR (ddPCR) analysis* is used to quantify circRNA copy number (Hindson et al., 2011). Compared to the conventional RT-qPCR, ddPCR is more accurate, provides absolute numbers, and measures low-abundance RNAs. However, it requires special instrumentation, software, and proprietary reagents (Quan, Sauzade, & Brouzes, 2018). Fifth, *northern blot analysis* is another technique used to investigate circRNAs size, isoforms, processing, sequence, and abundance, and also to distinguish between a circRNA and its linear counterpart (Pamudurti et al., 2017). Sixth, *RNA fluorescence in situ hybridization (RNA-FISH) coupled with high-resolution microscopy* is a commonly used technique to examine the abundance and localization of RNA molecules. RNA-FISH probe targets circRNA junctions to detect and quantify several circRNAs and determine the co-localization of circRNAs with proteins (Itzkovitz & van Oudenaarden, 2011; Jeck & Sharpless, 2014).

Furthermore, several online circRNA resources and public databases have been developed to provide key bioinformatics knowledge about circRNAs (Table 2B) including exoRBase (http://www.exorbase.org/) which was mentioned earlier in this review. *CircIntractome* can be used to design divergent primers and siRNA (Dudekula et al., 2016). C*ircBase* can provide information about circRNA identity and tissue specificity based on RNA-seq data and identify potential interacting factors such as miRNAs and RBPs (Memczak et al., 2013).

Since circRNAs have the ability to regulate gene expression, it exist as potential therapeutic tool for various diseases (Li, Yang, & Chen, 2018b). Currently, molecular tools for circRNA manipulation are under investigation. Furthermore, the manipulation of circRNA expression levels is another strategy to understand the impact of its expression levels in disease progression. CircRNA overexpression (gain of function) and knockdown (loss of function) approaches have been successfully used to study circRNAs. CircRNA gain of function approaches to overexpress circRNAs have been used to design expression vectors containing the circRNA sequence and delivered by viral vector systems such as plasmid transfection

and AAV vector (Hansen et al., 2013; Meganck et al., 2018) which already used in clinical trials (Jessup et al., 2011). The limitation of this technique is the formation of concatemers if the RNA polymerase cannot recognize the transcription terminator site (TTS) (Barrett & Salzman, 2016). Therefore, northern blot is required to exclude concatemer expression. Non-viral strategies are also developed to produce exogenous circRNAs *in vitro* in eukaryotic cells (Hansen et al., 2013; Wesselhoeft, Kowalski, & Anderson, 2018).

In addition, circRNA loss-of-function experiment is usually used to knockdown the circRNAs expression. Small interfering RNA (siRNA) and small hairpin RNA (shRNA) designed specifically to target the circRNA junction (back-splice site) without affecting the linear mRNA counterpart are used to successfully knockdown circRNA expression (Chen et al., 2017b). However, the limitation of this technique is that the siRNA may target the linear mRNA or other circRNAs with the same junction sequence (Holdt et al., 2016; Wang, Long, et al., 2016a). Therefore, the negative effect of siRNA-mediated knockdown on the parental gene expression should always be examined and ruled out. Also, CRISPR-Cas9 genome editing technology can be used to delete intronic complementary regions and potentially generate circRNA knockouts (Abudayyeh et al., 2017; Cox et al., 2017). This tool was used to remove the circRNA CDR1as locus from the mouse genome to generate CDR1as-loss-of-function mutant mice (Piwecka et al., 2017). Moreover, the CRISPR/Cas9 genome editing was used to remove the intronic complement sequence (ICS) of the circGCN1L1-flanking introns to suppress its expression without affecting the linear mRNA (Zhang et al., 2016). In general, manipulation tools for circRNA are a considerable gain in uncover circRNA function in diseases, and they have perfect prospects for being progressing into circRNA-based therapeutic strategies in the future.

8. Exosomal circRNAs

CircRNAs have a high degree of stability and resistance to exonuclease degradation; therefore, they may be accumulated in the cells if not controlled by cellular mechanism (Conn et al., 2015). One of the most important cellular mechanisms is the excretion of circRNAs from cells through extracellular vesicles such as exosomes. Exosomes are membrane-bound vesicles of endocytic origin secreted by most cell types (Xu et al., 2018; Yang et al., 2017b) and contain cellular components such as proteins, lipids, mRNAs, and miRNA (Valadi et al., 2007) and participate in cell-to-cell communication, and transfer genetic information (Bai et al., 2019). Recently, circRNAs have been detected in exosomes indicating that cells may use exosomes to transport circRNAs to communicate to other cells (Lasda & Parker, 2016). In 2015, *Li et al.* reported, for the first time, that exosomes contain abundant circRNAs (Lener et al., 2015). These circRNAs within the exosomes (referred to as exo-circRNAs) are stable and circulate in the serum after RNase R treatment. The presence of exo-circRNAs has been confirmed in a variety of cells. It has been reported that numerous exo-circRNAs could be released into extracellular space and detected in the blood, which is used as potential biomarkers in cancers. In addition, exo-circRNAs could enter the cell again (autocrine effect), taken by adjacent cell (paracrine effect), or enter a distant cell (endocrine effect) (Fig. 5) and release their content (Milman, Ginini, & Gil, 2019). However, the functions of exosomal circRNAs are not fully clear. By delivering circRNAs, exosomes play a key role in regulating signaling transduction between neighboring or distant cells and

transfer biological activities to recipient cells (Li, Zheng, et al., 2015a; Théry, Zitvogel, & Amigorena, 2002).

Furthermore, circRNAs have been reported to be able to bind to miRNAs, which are also shown to be abundant in exosomes (exomiRNAs) (Li, Zheng, et al., 2015a). CircHIPK3 has been reported to be transferred from cardiomyocytes into cardiac microvascular endothelial cells by exosomes and protect endothelial cells from oxidative damage and vascular dysfunction *in vitro* under oxidative stress conditions by sponging miR-29 (Wang et al., 2019b). Additionally, exosomal ncRNAs are involved in cardiovascular diseases and their potential use as disease biomarkers (Jaquenod De Giusti, Santalla, & Das, 2019). However, the roles of exo-circRNAs in accelerating and propagating cardiovascular and metabolic inflammation still require further study.

Currently, a database named exoRBase (http://www.exorbase.org/) is mainly a collection of all long RNA species derived from RNA-seq data analyses of human blood exosomes. It studied the RNA expression profiles in normal individuals and patients with different diseases. The first release of exoRBase contains 58,330 circRNAs, 15,501 lncRNAs and 18,333 mRNAs from 87 blood exosomal RNA-seq datasets. Compared to healthy individuals, patients with coronary heart disease (CHD) significantly upregulated 2,425 exo-circRNAs and downregulated 485 exo-circRNAs (Li, Li, et al., 2018a). These finding indicated that exo-circRNAs play a role in the development of the disease and can be used as disease biomarkers.

9. CircRNAs as potential diagnostic biomarkers

In the early stages of a disease, the characterization of biomarkers is considered a very promising strategy in the diagnosis and prevention of the disease. Since circRNAs are highly abundant, stable, and have tissue-specific expression, previous reports highlighted their potential as disease biomarkers. In addition to this, it has been shown that circRNAs are packaged and released in exosomes that provide additional protection to it, and increasing amounts were found in exosomes when compared to the cells (Li, Zheng, et al., 2015a). Previous studies have demonstrated that the circRNAs hsa_circ_002059 and hsa_circ_104916 were dramatically downregulated in the plasma and gastric cancer tissues and significantly correlated with tumor metastasis, age, and sex, suggesting the potential of these circRNAs as a stable diagnostic biomarker for gastric cancer (Li et al., 2015d; Li et al., 2017b). Additionally, circ-ITCH can be used to diagnose esophageal cancer (Li, Zhang, et al., 2015c), whereas hsa_circ_0005075 can be used as a potential biomarker of hepatocellular carcinoma (Qin et al., 2016). Although several circRNAs have been examined and suggested to exert important functions in the pathological process of various cardiovascular diseases (CVDs) (Fan et al., 2017), however, very few circRNA biomarker candidates for CVDs have been reported. The expression of circRNAs was investigated in peripheral blood of patients with coronary heart disease (CHD) and results suggested that circRNA hsa_circ_0124644 could be used as a diagnostic biomarker of CHD (Memczak et al., 2015; Zhao et al., 2017b). Another study reported that circulating circRNA hsa_circ_0021001 was decreased in peripheral blood of patients with intracranial aneurysms (IA), and was considered to be a potential diagnostic marker for IA (Teng et al., 2017).

Furthermore, bioinformatics analysis of peripheral blood circRNA expression profiles in hypertensive patients identified circRNA hsa-circ-0005870 as a novel biomarker for diagnosis of hypertension (Wu, Jin, & Cai, 2017). Another study identified circRNA MICRA as a novel prognostic biomarker for patients with heart failure after myocardial infarction (Salgado-Somoza, Zhang, Vausort, & Devaux, 2017). CircRNAs can also be used as diagnostic biomarkers in central nervous system (CNS). These circRNAs can cross the blood-brain barrier (BBB), enter into the blood and cerebrospinal fluid (CSF), and thus be used as novel biomarkers (Lu & Xu, 2016).

10. Regulation of oxidative stress and reactive oxygen species (ROS) production by circRNA

Oxidative stress is characterized by an imbalance between oxidants and antioxidants and a disruption of redox signaling (Sies, 2015). It is generally accepted that oxidative stress can lead to cell and tissue injury having a fundamental role in vascular dysfunction and inflammation (Siti, Kamisah, & Kamsiah, 2015; Steven et al., 2019). Under physiological conditions, ROS control vascular function by modulating various redox-sensitive signaling pathways. However, in vascular disorders, oxidative stress triggers endothelial dysfunction and inflammation, affecting several cells in the vascular wall including endothelial cells and vascular smooth muscle cells (Steven et al., 2019; Sun et al., 2020). Recently, we reported that mitochondrial ROS, uncoupled from ATP synthesis, determine endothelial cell activation status for both physiological recruitment of patrolling cells (at low ROS levels) and pathological recruitment of inflammatory cells (at high ROS levels) (Cheng et al., 2017; Li et al., 2013; Li et al., 2016b; Li et al., 2017c; Nanayakkara, Wang, & Yang, 2019). Several studies demonstrated that several circRNAs are regulated by oxidative stress and mediate ROS production as well as promote ROS induced cellular death, cell apoptosis, and inflammation (Cheng, Cao, Xue, Xia, & Xu, 2019; Li et al., 2020a; Liang et al., 2020). A previous study showed that sodium/calcium exchanger 1 (ncx1) circular RNA (circNCX1) was upregulated in response to ROS (H_2O_2) in cardiomyocytes by acting as a miR-133a-3p sponge to release the pro-apoptotic gene cell death inducing p53 target 1 (CDIP1) and promote cardiomyocyte apoptosis (Li et al., 2018c). However, H_2O_2 stimulation resulted in the downregulation of circHIPK3 expression in human osteoblast whereas circHIPK3 overexpression alleviated H_2O_2-induced cell viability reduction, cell death and apoptosis (Liang et al., 2020). Furthermore, autophagy-related circular RNA (circACR) is downregulated by high glucose (HG) irritation, and its overexpression attenuated HG-aroused SchwannRSC96 cell apoptosis, autophagy, and ROS generation (Liu, Chen, Yao, & Kang, 2019) (Table 3). Interestingly, circHIPK3 can be transferred by exosomes released from hypoxia-pretreated cardiomyocytes into cardiac microvascular endothelial cells, and protect endothelial cells from oxidative damage and vascular dysfunction *in vitro* under oxidative stress conditions by sponging miR-29a (Wang, Zhao, et al., 2019b). These studies indicated that circRNAs play a critical role in vascular pathology especially in disease conditions wherein oxidative stress plays a crucial role, such as endothelial dysfunction, vascular inflammation, diabetes mellitus, and atherosclerosis (Fuschi, Maimone, Gaetano, & Martelli, 2019).

11. Association of innate immune response with circRNA

The activation of the innate immune system results in enhanced responsiveness to subsequent triggers, which is termed trained immunity or innate immune memory (Netea & van der Meer, 2017). Not only the innate immune cells such as (monocytes, macrophages, and NK cells), but also traditional non-immune cells such as endothelial cells and smooth muscle cells have this memory function (Hamada, Torre, Drancourt, & Ghigo, 2018; Shao et al., 2020a). As discussed above, we recently proposed a new working model that endothelial cells are conditional innate immune cells (Wang, Zhao, et al., 2019b). Challenged cells with exogenous or endogenous insults results in metabolic reprogramming and epigenetic modifications (Lu et al., 2019), which is phenomenon seen in trained immunity of innate immune cells. Furthermore, when cells are exposed to a subsequent non-specific immune stimulus, they respond more strongly (secondary response) than to the primary insult (Netea, Quintin, & van der Meer, 2011; Zhong, Yang, Feng, & Yu, 2020). The metabolic reprogramming in trained immunity is characterized by increased glycolysis, increased acetyl-CoA generation, and increased cholesterol (mevalonate) pathway synthesis (Penkov, Mitroulis, Hajishengallis, & Chavakis, 2019). Initiation of innate immune memory through trained immunity is the likely mechanism behind the non-specific protective effects of certain vaccines (Benn, Netea, Selin, & Aaby, 2013), while increased inflammatory responsiveness of the cells due to trained immunity could also play a central role in inflammatory diseases (Bekkering, Joosten, van der Meer, Netea, & Riksen, 2013). We recently reported that pro-atherogenic lysophosphatidylcholine (LPC) activates human aortic endothelial cells (HAECs) (Li et al., 2018d; Li et al., 2018e; Li, Fang, et al., 2016b) and upregulates trained immunity pathways (TIPs) via analyzing our RNA-Seq data and histone 3 lysine 14 acetylation (H3K14ac)-chromatin immunoprecipitation (CHIP)-Seq data, both performed on HAEC treated with LPC (Lu et al., 2019). CircRNAs have been shown to play a critical role in glycolysis through sponging miRNAs, which represses the expression of several enzymes and transporters of glycolysis pathway (Yu et al., 2019). Some experimental studies reported that circMAT2B (Li et al., 2019b) and circ-PRMT5 (Ding, Guo, Deng, & Li, 2020) promote glycolysis, while knockdown of circAKT3 (Xu, Jiang, Wu, & Zhang, 2020), circCUX1 (Li et al., 2019c), and circDENND4C (Ren et al., 2019) inhibits glycolysis in cancer cells.

Microbial lipopolysaccharides (LPS) induce Toll-like receptor (TLR) pathways and NF-κ activation leading to modulation of genes involved in innate and adaptive immunity (Rosadini & Kagan, 2017). A previous study reported that the activation of TLR in mouse macrophages regulate the expression of circRasGEF1B. Additionally, circRasGEF1B could modulate the expression of intercellular adhesion molecule 1 (ICAM-1) as part of the LPS response, and that knockdown of this circRNA leads to a reduction in ICAM-1expression levels *in vitro* (Ng et al., 2016). Furthermore, innate immune response and trained immunity contribute to the development of atherosclerotic cardiovascular diseases (Hansson & Hermansson, 2011).

12. Regulation of vascular inflammation by circRNA

Endothelial dysfunction (Etwebi, Landesberg, Preston, Eguchi, & Scalia, 2018; He et al., 2016; Shao et al., 2014) and vascular inflammation are associated with several pathological processes such as inflammatory and ischemic cardiovascular disease as well as metabolic disorders (Carmeliet, 2003; Puro, Kohmoto, Fujita, Gardner, & Padovani-Claudio, 2016).We recently proposed a new working model that endothelial cells are conditional innate immune cells (Wang, Zhao, et al., 2019b), which are equipped with receptors including Toll-like receptors and inflammasome/caspase-1 (Li et al., 2016c; Li et al., 2017d; Shen et al., 2010; Wang et al., 2016b; Yang, Yin, & Wang, 2008; Yin et al., 2013) for danger/pathogen-associated molecular patterns (DAMPs/PAMPs) for sensing various DAMPs and conditional DAMPs such as pro-atherogenic lysophospholipids (Li et al., 2016d; Shao et al., 2017; Wang et al., 2016c), hyperlipidemia (Yin et al., 2015), hypoxia (Fu et al., 2017), uremic toxins (Ferrer et al., 2016; Monroy et al., 2015; Sun et al., 2018), LPS (Sha et al., 2015; Virtue et al., 2017), pro-inflammatory cytokines such as interleukin-7 (IL-17) (Mai et al., 2016) and anti-inflammatory cytokines including interleukin-35 (IL-35) (Li et al., 2012; Li, Fang, Yang, Wang, & Yang, 2017; Li, Shao, et al., 2018e) and interleukin-10 (IL-10) (Li et al., 2020b) as well as having innate immune memory (trained immunity) functions (Lu et al., 2019; Shao et al., 2020b; Zhong et al., 2020). Thus, targeting vascular endothelial dysfunction and inflammation has enormous therapeutic potential in the prevention and treatment of vascular complications. Several lines of evidence indicate that circRNAs are expressed in different cardiovascular diseases (Holdt et al., 2016; Wang, Long, et al., 2016a; Zheng et al., 2016) that are usually associated with endothelial dysfunction, vascular smooth muscle cell proliferation and migration, and vascular inflammation (Eelen, de Zeeuw, Simons, & Carmeliet, 2015; Sena, Pereira, & Seiça, 1832).

CircRNAs participate in cardiovascular disease via miRNA sponging and thus regulating their target genes to maintain homeostasis (Su & Lv, 2020). Some of these circRNAs (pro-inflammatory circRNAs) have been shown to promote endothelial dysfunction, vascular inflammation, and cardiovascular disease (Table 4A). Of note, circRNA from lncRNA ANRIL (antisense ncRNA in the INK4 locus) was positively correlated with INK4/ARF (Cdkn2a–Cdkn2b genes encoding three potent tumor suppressors, namely $p16^{Ink4a}$, $p19^{Arf}$ and $p15^{Ink4b}$) expression and atherosclerosis risk (Burd et al., 2010). Circ-Sirt1 binds to miR-132/212 and controls NF-kappaB activation to mediate inflammatory phenotypic switching of vascular smooth muscle cells (VSMCs) which play a fundamental role in neointimal formation and atherosclerosis (Kong et al., 2019). Also, circRNA CDR1as could regulate the miR-7 on its target gene expression and promote myocardial infarction via promoting cell apoptosis (Geng et al., 2016). In 2017, *Du et al.* discovered that circRNA circ-Foxo3 promotes cellular senescence and cardiac cell death (Du et al., 2017). Meanwhile, other circRNAs (anti-inflammatory circRNAs) have been shown to attenuate endothelial dysfunction and vascular inflammation (Table 4B). For instance, circRNA circ_0003204 was significantly upregulated in oxLDL-activated endothelial cells, and its silencing promoted endothelial cell proliferation and angiogenesis (Li, Ma, & Yu, 2017f). However, more pro-inflammatory circRNAs associated with cardiovascular diseases have been identified and characterized than the anti-inflammatory circRNAs. In general,

circRNAs are potentially involved in vascular dysfunction/inflammation; however, we recently reported that pro-atherogenic LPC induced circRNAs may contribute to homeostasis in LPS-induced HAEC activation (Li, Sun, et al., 2019a). Of note, the relevance of circRNAs to vascular inflammation and its molecular mechanism in cardiovascular diseases remains poorly characterized, and a better understanding of circRNA involvement in vascular-related inflammation and cardiovascular diseases will form a basis for the development of these circRNAs as biomarkers in the cardiovascular system. Additionally, a better understanding of circRNA may lead to discovery and the development of therapeutic agents as well as better patient prognosis.

13. Conclusion

CircRNAs are now a noteworthy area in the field of RNA. Several studies have demonstrated that circRNAs are an abundant, diverse, stable, and conserved class of RNA molecules, representing a new type of regulatory ncRNA. Nevertheless, their regulation and biological roles are not yet clearly understood, as well as their degradation, localization, and their involvement in disease pathogenesis. Recent studies have demonstrated that circRNAs can act as miRNAs sponges, RBP sponges, regulate transcription, or affect gene expression, and a growing body of evidence suggests that there might be other functions remaining to be revealed. Recently, a circRNA database has been constructed to provide tissue-specific circRNA expression profiles and circRNA-miRNA-gene regulatory networks. Considering the studies mentioned above, circRNAs might be useful therapeutic agents. Controlling the expression of circRNAs in specific tissues and cells of the body might yield greatly reduced endothelial dysfunction and vascular inflammation associated with cardiovascular and metabolic diseases. The studies mentioned above provide novel insights on the roles of circRNAs in regulating ROS, vascular inflammation associated with cardiovascular and metabolic diseases.

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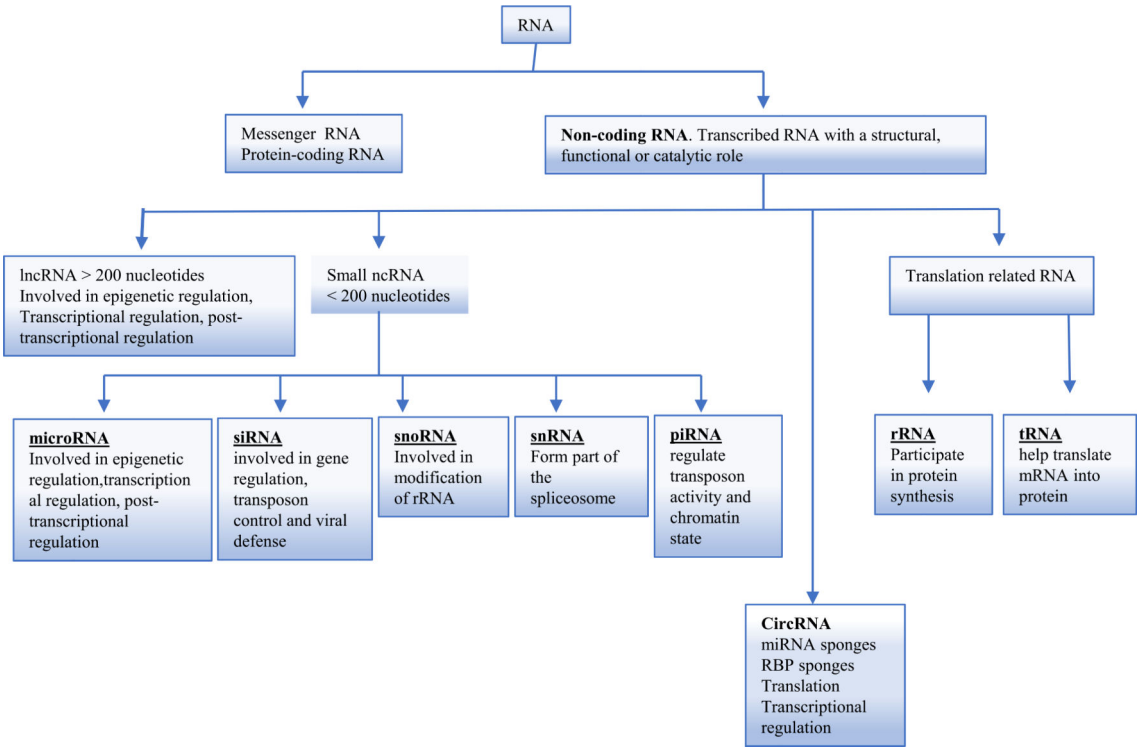


Fig. 1.

A classification of non-coding RNA and their biological functions. Abbreviations: ncRNA: non-coding RNA; circRNA: circular RNA; lncRNA: long non-coding RNA; miRNA: microRNA; siRNA: small interfering RNA; snoRNA: small nucleolar RNA; snRNA: small nuclear RNA; piRNA: piwi-interacting RNA; rRNA: ribosomal RNA; tRNA: transfer RNA.

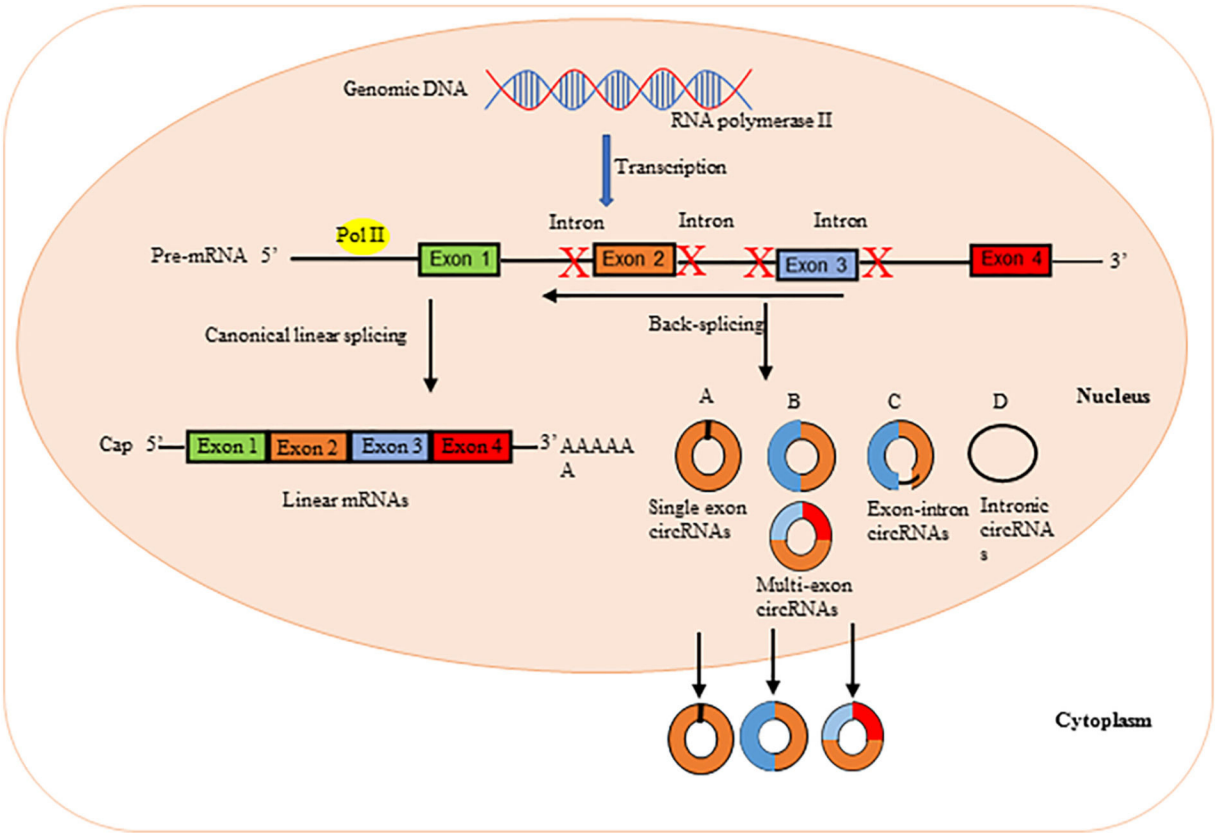


Fig. 2A.

Schematic representation illustrating circRNA biogenesis. In the nucleus of eukaryotic cells, DNA is transcribed to form precursor mRNA (pre-mRNA), which contain coding exons and introns. Different from linear mRNAs, which are formed by canonical linear splicing and cutting away introns of the pre-mRNAs using small nuclear ribonucleoproteins (snRNPs), circular RNAs (circRNAs) are formed by back-splicing of the pre-mRNAs and circularization of the cut segment, where the 5' end joins the 3' end. (A) Single exon circRNAs: circular RNAs can be generated from a single exon; (B) Multi-exon circRNAs: circular RNAs can also be generated from two or more exons; (C) Exon-intron circRNAs: circular RNAs can contain intron(s) that have been retained between one or more circular exons; (D) Intronic circRNAs: introns can be excised from pre-mRNAs and circularize to give rise to circRNAs.

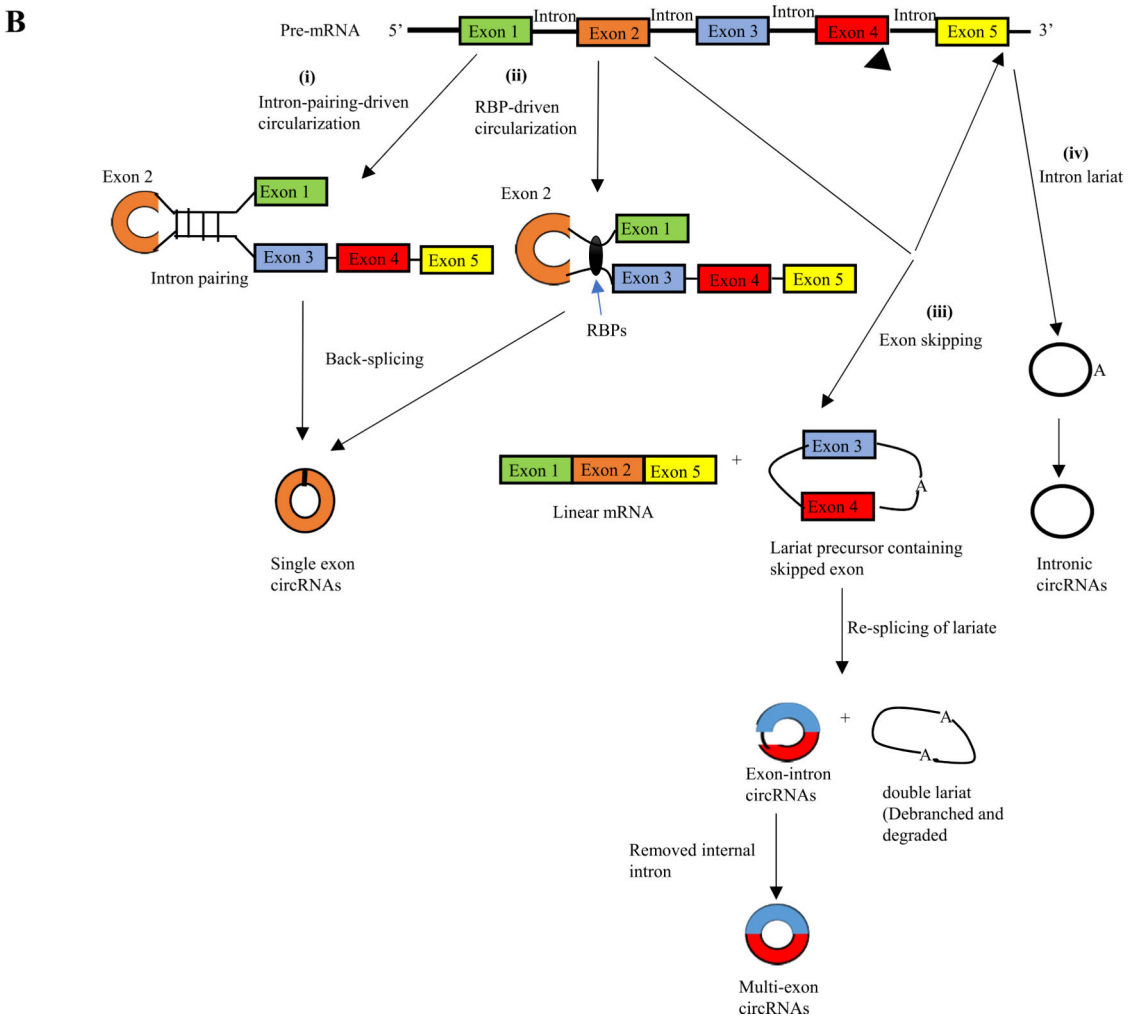
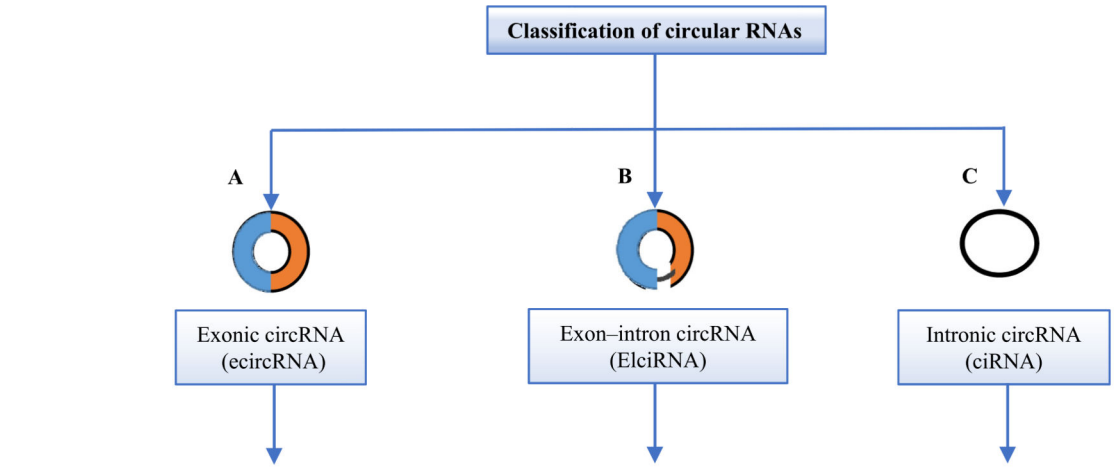


Fig. 2B.

The proposed models of circRNA formation. i) Intron-pairing-driven circularization. Two complementary introns form a circular structure containing several introns and exons through a base-pairing connection. Finally, introns are removed to form exonic circRNAs (EcircRNAs). ii) RNA binding protein (RBP)-driven circularization. The binding of RBPs acts as a vehicle that binds two non-adjacent introns. Then circRNAs are generated after the removal of introns. iii) Exon skipping: the back-splicing process can take place because of exon skipping mechanism, which leads to lariat formation. This process can generate three different products: linear mRNA, an exonic or exonic-interonic circRNAs, and intron lariats iv) Intron lariat will generate intronic circRNAs.



Location	Mainly cytoplasm	Mainly nucleus	Mainly nucleus
Origin	Derived from single or multiple exons	Derived from both exons and introns	Derived from single or multiple connected introns
Abundance	Largest class of circRNAs ~80%	Represent small fraction of circRNAs ~ 20%	Represent small fraction of circRNAs
Formation	Generated from back-splicing of pre-mRNAs by Lariat-driven circularization or intron-pairing driven circularization	Formed with introns 'retained' between exons during the back-splicing process by Lariat-driven circularization or intron-pairing driven circularization	Produced by intron splicing from lariat introns that can escape debranching
Function	MiRNA sponges; cis-regulation at post-transcriptional level; protein binding; encode functional proteins	Cis-regulation of parent gene	Cis-regulation of parent gene
Example	Cdr1as; circ-ITCH; hsa_circ_0031288	circEIF3J; circPAIP2	circ-ankrd52; circ-mcm5; circ-sirt7
PMID	27642589, 23446348	25664725	24035497

Fig. 3.

Three major subclasses of circRNAs. (A) Exonic circRNAs (ecircRNAs) consist of only exon(s) (usually less than five) and represent the most important group of circRNA class. EcircRNAs have cytoplasmatic location and may regulate microRNA and protein functions. (B) Exon-intron circRNAs (EIciRNAs) are composed of exons and retained introns. EIciRNAs have nuclear localization and have been found to be able to regulate gene transcription in *cis* and probably also in *trans*.(C) Intronic circRNAs (ciRNAs) are derived from intron lariats and are accumulated in the nucleus in which regulate gene transcription in *cis*.

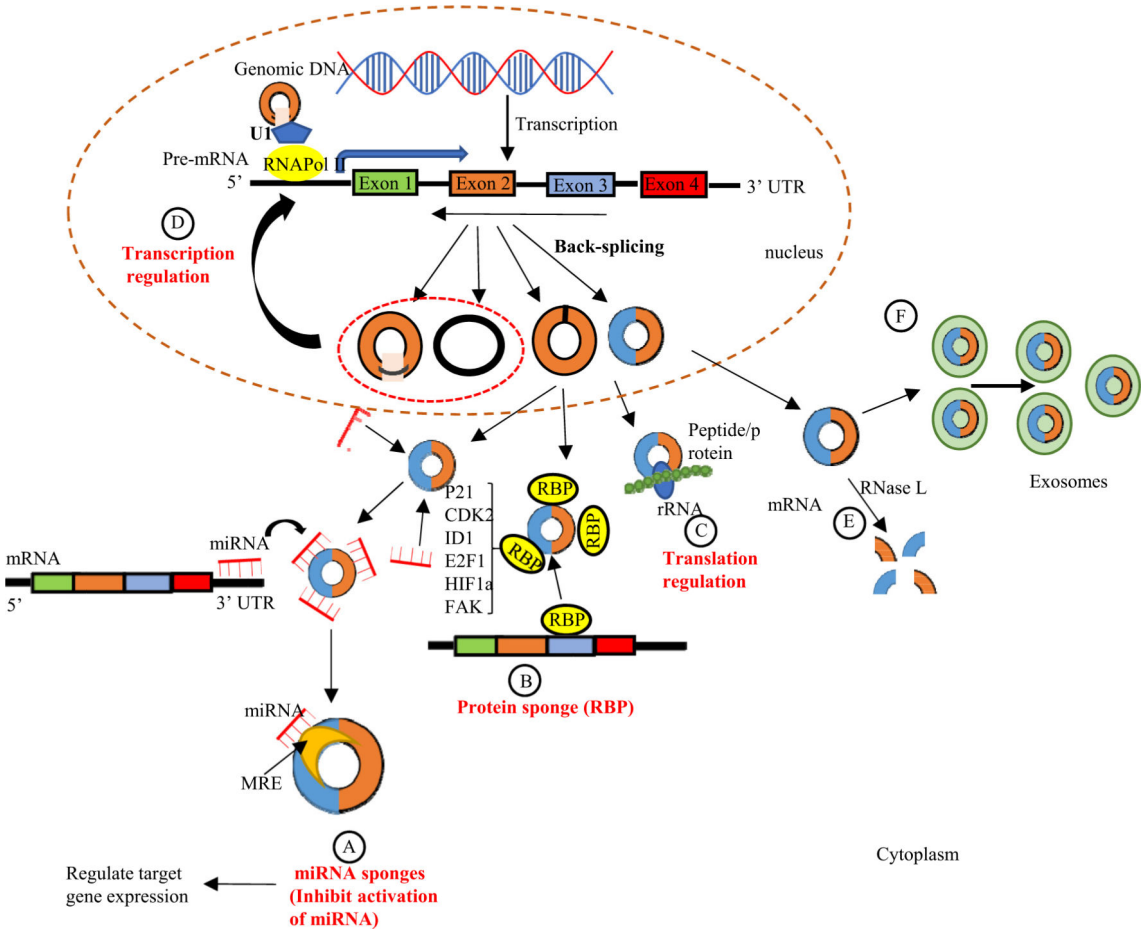


Fig. 4.

Schematic representation of circRNA functions and degradation. (A)microRNA sponges: circRNAs can act as miRNA sponges by competing for miRNA binding sites (miRNA response elements (MREs)) and prevents miRNA from interacting with their target messenger RNA (mRNA) at 3' untranslated region (UTR) leading to reduced the effect of miRNA-mediated regulatory activities.(B) Interaction with RNA binding proteins (RBPs): circRNAs may act as protein sponges, by directly binding to RBPs, and therefore retain them in the cytoplasm. These RBPs includes: cyclin-dependent kinase inhibitor 1 (p21), cyclin-dependent protein kinase 2 (CKD2), inhibitor of DNA binding 1 (ID1), E2F1, Hypoxia-inducible factor-1α (HIF1a), and preface focal adhesion kinase (FAK). (C) CircRNA can be translated with ribosome and encode peptides or proteins. (D) CircRNAs (e.g. EIciRNAs and ciRNAs) may interact with transcription complexes and enhance the expression of their parental genes. (E) The degradation of circRNAs. CircRNAs are globally degraded by RNase L in early cellular innate immune responses. (F) The elimination of circRNAs. circRNAs can be eliminated into the extracellular space by exosomes

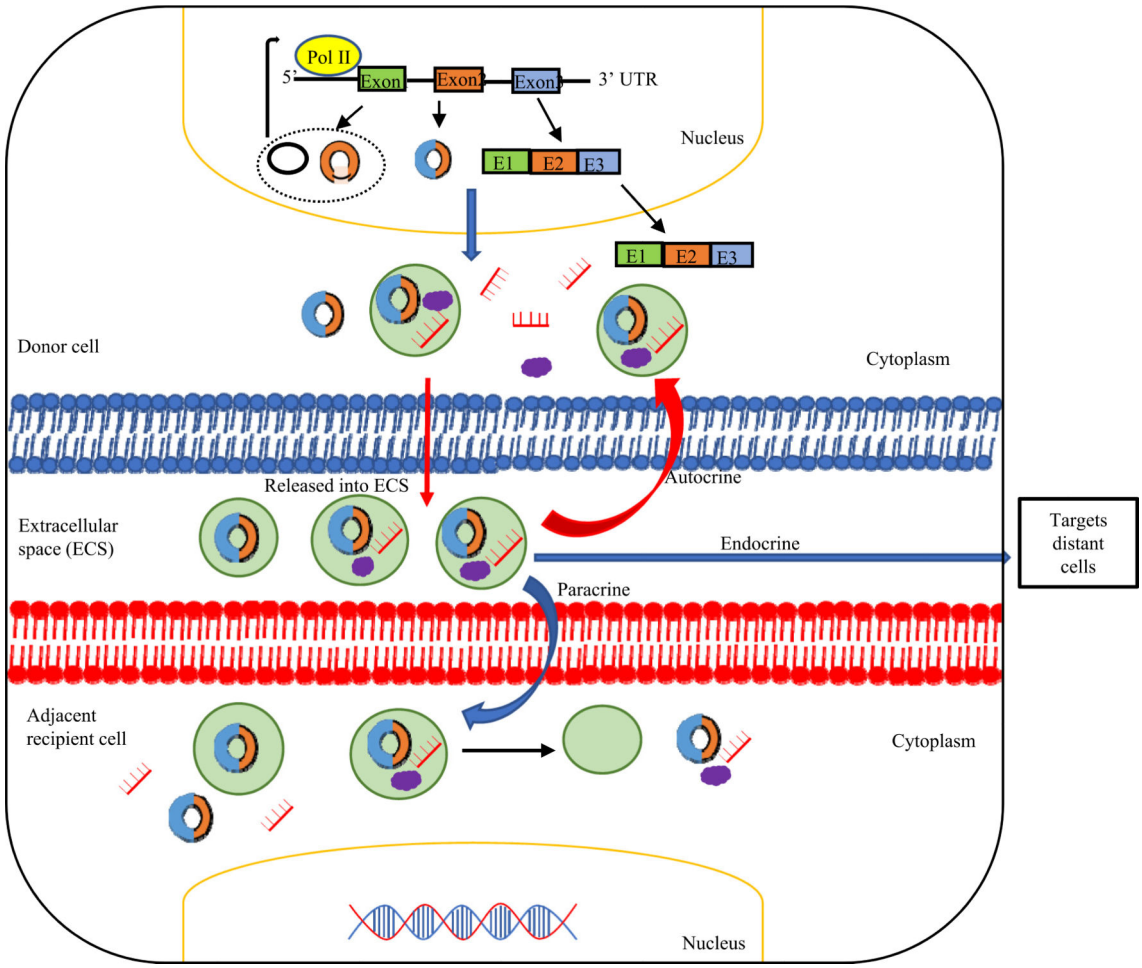


Fig. 5.

Generation of exosomal circRNAs (exo-circRNAs). circRNAs can be loaded into exosomes, released by donor cells into extracellular space, and enter the cells again (Autocrine effect) or targets nearby cells (Paracrine effect) or targets a distant cells (Endocrine effect) through endocytosis, thus modulating gene expression in recipient cells. Some exo-circRNAs are not bind to miRNAs in exosomes, they are able to sponge specific in target cells leading to target gene activation; or exo-circRNAs are bind to miRNAs in exosome. After entering target cells, miRNAs are released and target genes can be silenced.

Table 1

Three different types of non-coding RNAs and their features. Abbreviation: Pol II: polymerase II., pre-miRNAs: precursor miRNAs, PCR: polymerase chain reaction, RNA-FISH: RNA fluorescence in situ hybridization

Feature	CircRNA	miRNA	lncRNA
Origin	Originated from pre-mRNA by pol II via back-splicing	Originate from mRNAs transcripts to form primary miRNA and then undergo editing by adenosine deaminases and undergo processing by RNA Pol II Drosha in the nucleus to form pre-miRNAs then exported to cytoplasm and cleaved by Dicer to form mature double-stranded miRNAs	Transcribed from various genomic regions by both RNA Pol II and III
Length	>200 nucleotides	20 to 22 nucleotides	>200 nucleotides
Location	Nuclear and cytoplasm	Pre-miRNAs are found in the nucleus while mature miRNA found in the cytoplasm	Nuclear and cytoplasm
Degradation	Degraded by RNase L	Degraded by exoribonucleases such as small RNA degrading nuclease 1 (SDN1) and PNPase PNPT1	Degraded by RNase P cleavage
Conservation	Evolutionary conserved	Evolutionarily conserved	poorly conserved
Stability	Highly stable	Highly stable	Unstable
miRNA sponges	Yes	No	No
Splicing regulation	Yes	Yes	Yes
Interaction with RBPs	Yes	Yes	Yes
Transcriptional regulation	Yes	Yes	Yes
Post-transcriptional regulation	Yes	Yes	Yes
Translational regulation	Yes	Yes	Yes
Methods of detection	circRNA seq, microarray, PCR, digital droplet PCR (ddPCR), RNA-FISH, and northern blot analysis	RNA-seq, microarray, PCR, and RNA-FISH	RNA-seq, microarray, PCR, and RNA-FISH

Table 2A

Approaches to study circRNAs.

Method	Criteria	Unique feature	PMID
High-throughput circRNA sequencing (Circ-seq) analysis	Allows identification and annotation, high-throughput screening, and profiling under different conditions	Pre-treatment with RNase R is required to eliminate most linear transcripts while circRNAs are left intact Circ-seq analysis detects circRNA junctions.	28903484 27739534
CircRNA microarrays analysis	A useful tools for high-throughput analysis and multiple comparisons of the expression levels of specific circRNAs	Pre-treatment with RNase R is required to reduce the presence of linear RNAs and enhance circRNA detection and quantification	26451160 26484292
Reverse transcription-polymerase chain reaction (RT-PCR)	Used to validate the high-throughput screening Used to assess the relative abundance of the circRNAs Amplify speculative back-spliced junction (BSJ) sites but, this can amplify the linear RNA containing the same BSJ sequence locus and detects both circular and linear RNAs because of their sequence overlap.	Require the use of divergent primers that are designed to amplify and detect the BSJ, which can be designed using online resources such as CircInteractome Divergent primers will not amplify linear RNA Require the use of the RNA exonuclease enrichment strategy to digest all linear RNAs but does not digest circRNAs	16682442
Digital droplet PCR (ddPCR) analysis	A relatively new technique which used to quantify and measure the absolute levels of circRNAs	ddPCR is more accurate, provides absolute numbers, and measures low-abundance of circRNAs than RT-PCR	22035192
Northern blotting	Detect the abundances, sizes, isoforms, processing, and sequence of circRNAs. Can be employed to distinguish between a circRNA and its linear counterpart. The NB probes were labelled with the radioisotope 32P or non-radioactive probes labelled with digoxigenin.	This method is not suitable for high-throughput screening. A short probes can be employed that span the splice junction.	25921068
RNA fluorescence in situ hybridization (RNA-FISH)	Provide a tool for circRNAs visualization, subcellular localization, and quantification.	Require the use of RNA-FISH probes targeting circRNA back-spliced junctions to avoid recognition of the linear RNA counterpart. RNA FISH combined with RNase R treatment to impair linear RNA signals.	25664725 27050392
Online circRNA resources and databases	Allow the researchers to access the online circRNA databases. Examples of online databases (See table 1B)		
Overexpression of circRNAs	Gain of function analysis	This strategy used to design expression vectors for the ectopic expression of circRNAs and overexpress the brain-specific CDR1as and the testis-specific circSry	29174924 26450910 28344080
Depletion of circRNAs (knockdown)	Loss of function analysis Can be achieved by using circ siRNA/shRNA and CRISPR/Cas9-mediated genome editing The CircInteractome tool provides multiple options for circRNA siRNA	Designed specifically to target the circRNA junction without affecting the linear mRNA counterpart	21964070 18521077 25854182 27986464

Table 2B

Databases and online resources for circRNA research.

Database	Website	Note	PMID
CircBase	http://www.circbase.org/	Provides online data sets of circRNAs expression levels and sequence	25234927
CircInteractome	http://circinteractome.nia.nih.gov	Identification of RBPs and miRNAs binding sites on human circRNAs, design of divergent primers and siRNAs	26669964
circRNADb	http://reprod.njmu.edu.cn/circrnadb	Provides information on the circRNAs such as genomic information, genomic sequence, exon splicing, IRES, and open reading frames (ORFs)	27725737
exoRBase	http://www.exoRBase.org	Identify circRNAs in human blood exosomes. The first release of exoRBase contains 58,330 circRNAs	30053265
BBBomics	http://bioinformaticstools.mayo.edu/bbbomics/	CircRNA database specific for blood-brain barrier	26973449
CSCD	http://gb.whu.edu.cn/CSCD	A database for cancer-specific circRNAs that provides information on circRNAs expression, interacting miRNAs and RBPs	29036403
TSCD	http://gb.whu.edu.cn/TSCD	Provides a comprehensive characterization of tissue-specific circRNAs in the human and mouse genomes	27543790
CIRCpedia	http://www.picb.ac.cn/rnomics/circpedia	CircRNAs expressed in different species, cell types, and tissues	30172046
CircR2Disease	http://bioinfo.snnu.edu.cn/CircR2Disease/	Provides a comprehensive resource for circRNA deregulation in different diseases	29741596
TRcirc	http://www.licpathway.net/TRCirc	Provides a resource for transcriptional regulation information of circRNAs	30184150
CircView	http://gb.whu.edu.cn/CircView	Allow users to visualize circRNAs and view the regulatory elements, such as microRNA response elements and RNA-binding protein binding sites	29106456

Table 3

Circular RNAs regulates oxidative stress and reactive oxygen species (ROS). ROS including hydrogen peroxide (H_2O_2) has been shown to upregulate or downregulate circRNAs expression which either promote or attenuate the effect of ROS on the cell viability and apoptosis. Also, high glucose has been shown to affect circRNA expression and then regulate ROS production by acting as miRNAs sponge. Abbreviations: H_2O_2: Hydrogen peroxide; HG: High glucose; ROS: Reactive oxygen species; CircNCX1: Sodium/calcium exchanger 1 (ncx1) circular RNA; CircPRKCI: Protein kinase C iota type (PRKCI) circular RNA; CircHIPK3: Homeodomain-interacting protein kinase 3 (HIPK3) circular RNA; CircLRP6: Low-density lipoprotein receptor-related protein 6 (LRP6) circular RNA; CircACR: Autophagy-related circular RNA (ACR); HMGB1: High mobility group box 1; TLR4: Toll like receptor 4; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K: Phosphoinositide 3-kinase; AKT: Protein kinase B; mTOR: mammalian target of rapamycin

Stimulus	CircRNA	Expression of circRNAs	Function	Target	PMID
H_2O_2	CircNCX1	Upregulated by H_2O_2	Promote cardiomyocyte apoptosis by acting as an endogenous miR-133a-3p sponge and suppress the activity of pro-apoptotic gene cell death-inducing protein (CDIP1)	miR-133a-3p	30613267
H_2O_2	CircRNA-4099	Upregulated by H_2O_2	Overexpression of circRNA-4099 aggravated H_2O_2-induced cell apoptosis, ROS production and cell fibrosis	miR-706 and trigger keap1/ Nrf2 and p38MAPK	31479678
H_2O_2	CircPRKCI	Downregulated by H_2O_2	Overexpression of circPRKC1 attenuated H_2O_2-induced neuronal cell death and apoptosis	miR-545 and miR-589	31053300
H_2O_2	CircHIPK3	Downregulated by H_2O_2	Overexpression of circHIPK3 in human osteoblasts alleviated H_2O_2-induced viability reduction, cell death and apoptosis	miR-124	31955154
HG	CircLRP6	Upregulated by HG	Regulate HG-induced proliferation, oxidative stress, ECM accumulation, and inflammation in mesangial cells by upregulating HMGB1 and activating TLR4/NF-κB signaling	miR-205	31087368
HG	CircACR	Downregulated by HG	Overexpression of circACR relieved HG-aroused SchwannRSC96 cell apoptosis, autophagy and ROS generation	miR-145–3p and inhibit PI3K/AKT/mTOR pathway	31886589

Table 4A

Summary of identified Pro-inflammatory circRNAs. Different circRNAs that promotes vascular inflammation and cardiovascular diseases. Abbreviations: AS: Atherosclerosis; AIS: Acute ischemic stroke; CHD: coronary heart disease; HUVECs: human umbilical vein endothelial cells; VEC: vascular endothelial cells; VSMCs: vascular smooth muscle cells; Mø: macrophages; PBMC: peripheral blood mononuclear cells; Ox-LDL: oxidized low- density lipoprotein; LPS: lipopolysaccharides; Circ-RELL1: circular RNA-receptor expressed in lymphoid tissues-like protein 1 (RELL1); CircANRIL: circular antisense non-coding RNA at the INK4 locus (cANRIL); CircRNA HECTD1: circular RNA HECT domain E3 ubiquitin protein ligase 1.

CircRNAsUp	Expression of circRNAs	Cells	Function	Mechanism/ target	Pathways	PMID
circ-RELL1	Upregulated by ox-LDL	HUVECs	Increased endothelial inflammation in ox-LDL-stimulated HUVECs	miR-6873–3p	miR-6873–3p/ MyD88/NF-κB pathway	32113679
RNA-ZNF609	Upregulated in high glucose conditions, hypoxia stress and in diabetic retina	Retinal ECs	Aggravated oxidative stress/hypoxic stress-induced HUVEC apoptosis increased endothelial dysfunction	miR-615–5p	cZNF609/miR-615–5p/ MEF2A	28824721
hsa_circ_0068087	Upregulated in high glucose conditions	HUVECs	Accelerate TLR4/NF-κB/NLRP3 inflammasome-mediated inflammation and EC dysfunction in the high glucose condition	miR-197	TLR4/NF-κB/NLRP3 inflammasome	31108165
CircHIPK3	Upregulated in diabetic retina	Retinal VECs	Increased retinal VECs dysfunction/ inflammation	miR-30a-3p	circHIPK3-miR-30a-3p-VEGFC/WNT2/FZD4 pathway	28860123
CircANRIL	Overexpression in AS model	VECs	Increased VECs inflammation and AS	-	-	28683453
Circ_0003575	Upregulated by ox-LDL	HUVECs	Attenuated EC proliferation and angiogenesis	-	-	28946214
CircRNA-0044073	Overexpression in AS	VSMCs/VECs/ blood cells	Promoted the proliferation of VSMCs and HUVECs Promotes inflammation in AS	miR-107	JAK/STAT signaling pathway	30864721
Circ_CHFR	Upregulated by ox-LDL	VSMCs, Plasma	Promoted the ox-LDL-induced cell proliferation, migration, and inflammation	miR-214–3p	Wnt3/β-catenin signal pathway	32271446
CircWDR77	Upregulated in high glucose conditions	VSMCs	Induced VSMCs proliferation and migration	miR-124	CircWDR77-miR-124-FGF2 pathway	29042195
Circ_Lrp6	AS	VSMCs	Induced VSMCs proliferation and migration, and neointima hyperplasia; proatherogenic	miR-145	-	30582454, 30763218
hsa_circ_0029589 (circCHFR)	Upregulated by ox-LDL and in AS	VSMCs	Promotes VSMCs migration and proliferation	miR-370	miR-370/FOXO1/ Cyclin D1 pathway	31048182

Table 4B

Anti-inflammatory circRNAs. Different circRNAs that attenuates vascular inflammation and cardiovascular diseases. Abbreviation: HUVEC: human umbilical vein endothelial cells; AS: atherosclerosis; VSMCs: vascular smooth muscle cells; Mø: macrophages; HAECs: human aortic endothelial cells; Ox-LDL: oxidized low-density lipoprotein; TNF-α: tumor necrosis factor-alpha;

CircRNA name	Expression of circRNAs	Cells/tissue	Function	Mechanism/Target	Pathways	PMID
hsa_circRNA-0054633	Upregulated in high glucose conditions	HUVEC	Decreased the high glucose-induced endothelial cell dysfunction, including proliferation, migration and promote angiopoiesis	microRNA-218	microRNA-218/heme oxygenase-1 axes	29693114
CircANRIL	Downregulated in AS	VSMCs/Mø	Atheroprotection against human AS	Binds to PES1, thereby impairing pre-rRNA processing and ribosome biogenesis	-	27539542
Circ_0003204	Upregulated by ox-LDL and in cerebral AS	HAECs	Inhibits proliferation, migration and tube formation of ECs in AS	miR-370	miR-370–3p/TGFβR2/phosph-SMAD3 pathway	31900142
Circ-Sirt1	Downregulated by TNF-α stimulation and in neointima hyperplasia	VSMCs	Inhibits inflammatory phenotypic switching of VSMCs	miR-132/212	circ-Sirt1/miR-132/212/NF-κB pathway	30820544