Mini-review

Circular RNA: A new star of noncoding RNAs

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A B S T R A C T

Circular RNAs (circRNAs) are a novel type of RNA that, unlike linear RNAs, form a covalently closed continuous loop and are highly represented in the eukaryotic transcriptome. Recent studies have discovered thousands of endogenous circRNAs in mammalian cells. CircRNAs are largely generated from exonic or intronic sequences, and reverse complementary sequences or RNA-binding proteins (RBPs) are necessary for circRNA biogenesis. The majority of circRNAs are conserved across species, are stable and resistant to RNase R, and often exhibit tissue/developmental-stage-specific expression. Recent research has revealed that circRNAs can function as microRNA (miRNA) sponges, regulators of splicing and transcription, and modifiers of parental gene expression. Emerging evidence indicates that circRNAs might play important roles in atherosclerotic vascular disease risk, neurological disorders, prion diseases, and cancer; exhibit aberrant expression in colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC); and serve as diagnostic or predictive biomarkers of some diseases. Similar to miRNAs and long noncoding RNAs (lncRNAs), circRNAs are becoming a new research hotspot in the field of RNA and could be widely involved in the processes of life. Herein, we review the formation and properties of circRNAs, their functions, and their potential significance in disease.

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Introduction

Circular RNAs (circRNAs) were recently discovered as a special novel type of endogenous noncoding RNA and represent a recent research hotspot in the field of RNA. Unlike linear RNAs that are terminated with 5’ caps and 3’ tails, circRNAs form covalently closed loop structures with neither 5’–3’ polarities nor polyadenylated tails [1].

CircRNA was first found in RNA viruses as early as the 1970s [2]. Unfortunately, only a handful of such circRNAs were serendipitously discovered over the past 30 years [3–9]. Such molecules were typically considered to be molecular flukes or products of aberrant RNA splicing due to their low levels of expression. However, with the development of RNA deep sequencing technology and bioinformatics, recent work has revealed that large numbers of circRNAs are endogenous, abundant, conserved and stable in mammalian cells [10–16]. Furthermore, several researchers have confirmed that reverse complementary sequences including inverted repeated Alu pairs (IRAlus) and exon skipping are essential to circRNA formation [17–25]. Moreover, RNA-binding proteins (RBPs) also regulate circRNA formation [23,26].

Specifically, subsequent reports revealed that circRNAs could function as microRNA (miRNA) sponges, regulate alternative splicing, and modulate the expression of parental genes [13,14,16,23,27]. More importantly, it is becoming evident that circRNAs may be involved in atherosclerotic vascular disease risk, neurological disorders, prion diseases and cancer [28–30]; are aberrantly expressed in colorectal cancer (CRC) [31] and pancreatic ductal adenocarcinoma (PDAC) (S.B.Q., unpublished observations). CircRNAs were described as potential disease biomarkers in human saliva and as biomarkers for aging and gastric cancer (GC) [32–34]. Taken together, these findings indicate that circRNAs have great potential to perform special regulating roles in biological development and disease initiation and progression, become new clinical diagnostic and prognostic markers, and provide new insights into the treatment of diseases.

In this review, we briefly delineate the diversity of circRNAs and discuss the highlights of the biogenesis of circRNAs, their characteristics, their potential functions and their relationships with the disease.

Diversity of circRNAs

CircRNAs are expressed at low levels and were originally thought to be by-products of spliceosome-mediated splicing errors [35] or intermediates that escaped from intron lariat debranching [36,37]. Thus, circRNAs received little attention and were thought to be
unlikely to play critical roles in biological processes. Until 2010, few circRNAs had been discovered, and research into circRNA biogenesis was minimal. However, with the development of high-throughput sequencing technology and computational analysis, thousands of circRNAs across species from Archaea to humans have been discovered [10–16,38]. The expression of some circRNAs is >10-fold higher than those of their canonical linear transcripts of the same genes [12]. The recently identified human circRNAs are depicted in Table 1.

Biogenesis of circRNAs

Recent studies have revealed that the biogenesis of circRNAs via backsplicing is different from the canonical splicing of linear RNAs [18]. Furthermore, several recent advances in our understanding of circRNA biogenesis, particularly regarding its regulation and the competition between backsplicing and canonical splicing, have been made [1]. For example, Jeck et al. put forward two models of circRNA formation [12]. Model 1 is termed ‘lariat-driven circularization’ or ‘exon skipping’ (Fig. 1a), and model 2 is termed ‘intron-pairing-driven circularization’ or ‘direct backsplicing’ (Fig. 1b). Notably, Kelly and colleagues also found that exon circularization is widespread and correlated with exon skipping in human umbilical vein endothelial cells (HUVECs) treated with tumor necrosis factor α (TNFα) or tumor growth factor β (TGFβ) [22]. Although some evidence has indicated that intron-pairing-driven circularization might occur more frequently than lariat-driven circularization [39], accumulated evidence has verified the model of intron-pairing-driven circularization and suggested that reverse complementary sequences, including IRAlus, are important for circRNA biogenesis [17–21,23–25]. Shortly thereafter, Zhang and others discovered a new type of circRNA in human cells that is derived from introns and was termed circular intronic RNAs (ciRNAs). ciRNA biogenesis depends on a consensus motif containing a 7–nt GU-rich element near the 5′ splice site and an 11–nt C-rich element near the branchpoint site [14] (Fig. 1c). Very recently, Li et al. also found exons that are circularized with introns ‘retained’ between the exons. These authors termed them exon-intron circRNAs or ElciRNAs and found that they could be overexpressed with their flanking complementary sequences [16]. However, the mechanism of ElciRNA formation remains unknown. These mechanisms add considerably to the regulatory complexity of the human transcriptome.

Additionally, researchers have identified the muscleblind protein (MBL), which can bind to circMBL flanking introns to provoke the formation of circRNAs that act as RBPs to bridge two flanking introns close together [23]. Similarly, researchers reported an additional mode of circRNA biogenesis in which interactions between RBPs form a bridge between the flanking introns, which causes the splice donor and splice acceptor to close to promote circRNA biogenesis [40] (Fig. 1d). Surprisingly, Conn and others have recently found that RBP Quaking (QKI) regulates the formation of circRNAs [26]. In contrast, Ivanov and others noted that the RNA-editing enzyme ADAR1 can bind to double-stranded RNA to antagonize circRNA biogenesis by melting the stem structure [20]. Therefore, RBPs may serve as activators or inhibitors of the formation of circRNAs in some conditions.

Remarkably, Zhang et al. first proposed a model of alternative circularization that is similar to alternative splicing [18] (Fig. 2). These authors found that competition in RNA pairing by complementary sequences (either repetitive or nonrepetitive) across or within individual flanking introns could significantly affect splicing selection and exon circularization. Complementary sequences within individual flanking introns can be sufficient to promote longer mRNA generation. Conversely, complementary sequences across flanking introns can benefit exon circularization. The competition between reverse complementary sequences can result in multiple circRNA transcripts being processed from a single gene (Fig. 2). However, alternative circularization can be species-specific due to the different distributions of complementary sequences across species. The existence of complementary sequences is necessary but not sufficient for exon circularization [18]. This model suggests that the mechanism of alternative circularization is very complicated and is also possibly regulated by other factors, such as RBPs [1].

Properties of circRNAs

According to recent research, there are several noteworthy properties of circRNAs that are produced by backsplicing. Firstly, these circRNAs have covalently closed loop structures with neither 5′–3′ polarity nor a polyadenylated tail, which makes them much more stable than linear RNA and susceptible to degradation by RNA exonuclease or RNase R [41]. For example, researchers identified >400 circRNAs in human cell-free saliva (CFS) from healthy individuals. These data represent experimental validation of circRNAs in any type of extracellular body fluid [33]. Secondly, there is a great diversity of circRNAs [40]. In some cases, the abundance of circular molecules exceeds those of the corresponding linear miRNAs by >10-fold [12]. Thirdly, circRNAs are largely composed of exons, which primarily reside in the cytoplasm and possibly have miRNA response elements (MREs) [11–13]. Moreover, circRNAs harbor significant reductions in polymorphisms at predicted miRNA target sites [42]. Some circRNAs come from introns or exons with introns that are ‘retained’ between exons and are primarily located in the nucleus in eukaryotes and may regulate gene expression [14,16].

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**Table 1**

Overview of human circRNAs identified recently.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Special treatment</th>
<th>Detection method</th>
<th>Number of circRNAs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line (HeLa)</td>
<td>Pol II CLIP</td>
<td>RNA-seq</td>
<td>15 ElciRNAs (most abundant)</td>
<td></td>
</tr>
<tr>
<td>39 ENCODE data sets</td>
<td>rRNA depletion</td>
<td>RNA-seq</td>
<td>7112 predicted circRNAs (circRNA fraction ≥10%)</td>
<td></td>
</tr>
<tr>
<td>Cell line (H9)</td>
<td>poly(A) RNA depletion</td>
<td>RNA-seq</td>
<td>103 ciRNAs (at least 2-fold enrichment)</td>
<td></td>
</tr>
<tr>
<td>Cell line (H68)</td>
<td>rRNA depletion</td>
<td>RNA-seq</td>
<td>25,166 predicted circRNAs (high-confidence)</td>
<td></td>
</tr>
<tr>
<td>15 Cell lines (including cancer and non-cancer cell lines from public ENCODE RNA-seq data)</td>
<td>rRNA depletion</td>
<td>RNA-seq</td>
<td>46,866 predicted circRNAs (at an FDR of 0.025)</td>
<td></td>
</tr>
<tr>
<td>4 Cell lines (CD19+ leukocytes, HeLa, HKB293, CD34+ leukocytes, neutrophils)</td>
<td>RNase R</td>
<td>RNA-seq</td>
<td>1590 predicted circRNAs (at least two independent reads)</td>
<td></td>
</tr>
<tr>
<td>5 Cell lines (CD19+ leukocytes, HeLa, H9, CD34+ leukocytes, neutrophils)</td>
<td>rRNA depletion</td>
<td>RNA-seq</td>
<td>2748 predicted circRNAs</td>
<td></td>
</tr>
</tbody>
</table>

Special treatments were conducted after total RNAs were extracted from the samples. Then, circRNAs were identified via RNA-seq. circRNAs: circular RNAs; ElciRNAs: exon–intron circRNAs; ciRNAs: circular intronic RNAs; rRNA: ribosomal RNA; RNA-seq: RNA-sequencing; Pol II CLIP: RNA polymerase II crosslinking and immunoprecipitation; FDR: false discovery rate; RNase R: ribonuclease R.

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Fourthly, circRNAs often exhibit tissue/developmental-stage-specific expression \[11,13,16\]. For example, hsa_circRNA_2149 has been detected in CD19\(^+\) leukocytes but not CD34\(^+\) leukocytes, neutrophils or HEK293 cells. Some nematode circRNAs seem to be expressed in oocytes but absent in 1- or 2-cell embryos according to sequencing data \[13\]. Fifthly, the vast majority of circRNAs are endogenous noncoding RNAs, and only a small portion of exogenous circRNAs, such as Hepatitis \(\delta\) (HDV) and engineered circRNAs with internal ribosome entry sites (IRESs), are translated \[5,43,44\]. Finally, circRNAs are evolutionarily conserved between different species \[11,12,45\]; however, some ciRNAs are much less evolutionarily conserved \[14\]. Taken together, these properties indicate that circRNAs have the potential to play important roles in transcription and post-transcription and to become ideal biomarkers in the diagnosis of diseases.

CircRNA function

CircRNAs function as competing endogenous RNAs or miRNA sponges

The competitive endogenous RNAs (ceRNAs) contain shared MREs, such as mRNAs, pseudogenes and long noncoding RNAs (lncRNAs), and can compete for miRNA binding \[46\]. Thus, the presence or absence of ceRNAs influences the activities of miRNAs regarding the
regulation of gene expression. Recently accumulated evidence indicates that circRNAs can function as miRNA sponges or potent ceRNA molecules [13,27,30,47] (Fig. 3) and can be depleted of polymorphisms at microRNA binding sites [41]. For example, the exonic circRNAs ciRS-7/CDR1as (for circular RNA sponge for miR-7 or CDR1 antisense) and Sry have been shown to bind miRNAs without being degraded, which makes them excellent candidates for ceRNA activity [39]. Hansen et al. discovered that the cerebellar degeneration-related protein 1 (CDR1) gene can translate a natural circular antisense transcript termed antisense to the cerebellar degeneration-related protein 1 transcript (CDR1as). CDR1as can interact with miRNAs and be cleaved by miR-671 [48]. The miR-671 binding site exhibits near-perfect complementarity and little variation across species [27]. Subsequent research revealed that CDR1as contains over 70 conserved seed matches for miR-7 and is densely bound by Argonaute proteins (i.e., the proteins that bind to miRNAs). Notably, the limits in the complementarity of the seed matches protect CDR1as from degradation from the bound miR-7 [27]. The silencing of CDR1as or the overexpression of miR-671 decreases the expression of published miR-7 targets [13,27]. In comparison, CDR1as overexpression prevents the downregulation of miR-7 targets [27]. Moreover, CDR1as is expressed at higher levels in nervous tissue. The overexpression of CDR1as in zebrafish embryos, which lack the cdr1 locus, substantially reduces midbrain size and mimics the phenotype of miR-7 loss-of-function, which causes morphological defects in the midbrain [13]. Similarly, Murine Sex-determining region Y (Sry) is the gene responsible for mammalian sex determination and can produce a testis-specific circular transcript [7]. This single-exon circRNA has 16 binding sites for miR-138 and can be co-precipitated with Argonaute 2 (AGO2) in HEK293 cells that are co-transfected with the circRNA Sry expression vector and pJEBB-138. These data indicate that the circular Sry RNA likely also acts as a miR-138 sponge [27].

However, some analyses of the large set of exonic circRNAs identified by CircleSeq suggest that very few circRNAs in mammalian
cells contain more than ten binding sites for an individual miRNA. Furthermore, many exonic circRNAs only contain smaller numbers of putative miRNA binding sites [39]. Analogously, Guo et al. also found that few circRNAs exhibit properties expected of miRNA sponges [15]. Fortunately, Li et al. detected that cir-ITCH spans several exons of the E3 ubiquitin (Ub) protein ligase (ITCH) and acts as a sponge of miR-7, miR-17 and miR-214 [30]. Therefore, whether circular miRNA sponges are a general phenomenon and how networks of circRNAs, miRNAs and ceRNAs maintain balance to regulate cellular homeostasis remain to be clarified.

CircRNAs regulate alternative splicing or transcription

Previous studies have suggested that circRNAs are involved in the regulation of alternative splicing or transcription. For example, Ashwal-Flusser et al. discovered that circMbl is generated by the second exon of the splicing factor MBL, which competes with canonical pre-mRNA splicing. circMbl flanking introns and circMbl itself have conserved MBL binding sites that are strongly and specifically bound by MBL. The modulation of MBL levels significantly affects circMbl formation, and this effect depends on MBL binding sites in the flanking intronic sequences [23]. These findings suggest that general splicing factors, such as MBL, may have effects on alternative splicing that modulate the balance between circRNA biogenesis and canonical splicing. Moreover, Chao et al. noticed that the mouse formin (Fmn) gene can produce circRNA via backsplicing. Notably, this circRNA that contains the translation start site functions as an ‘miRNA trap’ and leaves a noncoding linear transcript and thereby reduces the expression level of the Fmn protein [52]. Moreover, Jeck and Sharpless uncovered that many of single-exon circRNAs contain a translation start site in human fibroblasts [39]. These discoveries indicate that circRNAs could act as miRNA traps by sequestering the translation start site to regulate protein expression.

CircRNAs regulate the expression of parental gene

Recent advances have revealed that circRNAs could regulate the expression of parental genes (Fig. 4). For instance, Zhang and colleagues discovered that the formation of cirRNAs depends on the key flanking RNA elements that might be essential for the intron lariat to escape from debranching. These cirRNAs have little enrichment for microRNA target sites, indicating that they are functionally distinct [53]. Detailed studies have demonstrated that some cirRNAs are abundant in the nucleus and interact with the polymerase II (Pol II) machinery and modulate host transcription activity in a cis-acting manner [14]. Subsequently, researchers also reported a special class of circRNAs termed ElciRNAs that are associated with RNA Pol II in human cells. ElciRNAs, such as circEIF3J and circPAIP2, are predominantly localized to the nucleus, interact with U1 small nuclear ribonucleoproteins (snRNPs) and enhance the transcription of their parental genes in a cis-acting manner [16]. Similarly, Li and others found that both cir-ITCH and the 3′-untranslated region (UTR) of ITCH share some miRNA binding sites. Further study indicated that the interactions of cir-ITCH with miR-7, miR-17, and miR-214 might increase the level of ITCH [30]. We speculate that exon-only circRNAs may fulfill regulatory functions in the cytoplasm, whereas intronic circRNAs, such as cirRNAs and ElciRNAs, seem to be efficient for transcriptional regulation in the nucleus.
Other possible functions of circRNAs

Few circRNAs can be translated. Researchers reported that engineered circRNAs that were inserted an IRES in upstream of the start codons of a protein could be translated in vitro [42] or in vivo [54]. Similarly, Perriman and Ares reported that an engineered circular mRNA containing a single green fluorescent protein (GFP) open reading frame can direct GFP expression in Escherichia coli [44]. Interestingly, thus far, only one naturally occurring circRNA is known to encode a single protein in eukaryotic cells, i.e., HDV, which is a subviral satellite virus of the hepatitis B virus (HBV) [5]. The encapsulation of HDV with HBV virions results in the production of a single viral protein that is specific to pathogenicity, but the principle of translation is noncanonical and probably associated with specific viral agents [55,56]. However, to date, there is no evidence that suggests that naturally occurring endogenous circRNAs undergo translation [10,12]. Additionally, researchers have reported putative additional plausible roles of circRNAs [57,58]. For example, circRNAs could function as RBP sponges, e.g., the strong and direct interaction between MBL protein and circMbl [23] or function in the assembly of RBP factories or their allosteric regulators. CircRNAs could also directly target mRNAs by partial base pairing. Some circRNAs even serve as templates for translation, as indicated by findings that synthetic circRNAs can be efficiently translated. These findings demonstrate that further studies are necessary to clarify the other potential functions of circRNAs.

CircRNAs in disease

Recent works have suggested that circRNAs may play important roles in the initiation and development of disease could potentially become new biomarkers for these processes. For instance, the expression of cIRS-7/CDR1as but not CDR1 is induced by stable overexpression of the prion protein (PrPC) in HEK293 cells [59]. Therefore, PrPC could possibly be involved in the regulation of CDR1as. It would be interesting to unveil the function of CDR1as in prion disease [29].

CircMbl and its flanking intron sequences can be combined with MBL. Alterations in MBL levels strongly affect circMbl biosynthesis. circRNA production competes with canonical mbl pre-mRNA splicing [23]. MBL can regulate mbl pre-mRNA splicing efficiency between mbl mRNA and circMbl. Moreover, circMbl can sponge out the excess MBL protein by binding to it. However, MBL functional deficiency is known to cause a severe degenerative disease called myotonic dystrophy. Hence, we speculate that circMbl could be involved in myotonic dystrophy initiation and progression.

It is clear that miRNAs have been shown to be involved in nearly all aspects of cellular functions [60] and play critical roles in disease initiation and progression [61,62]. Given that circRNAs interact with miRNAs to regulate their target genes, circRNAs could possibly be involved in diseases correlated with miRNAs. For example, it is evident that CDR1as is highly expressed in the brain and has over 60 binding sites for miR-7 [13,27,48,63]. It is important to note that miR-7 is implicated in numerous pathways and diseases, including its function as a direct regulator of α-synuclein and ubiquitin protein ligase A (UBE2A). CDR1as has been implicated in Parkinson’s disease, Alzheimer’s disease and brain development [13,27,29,64]. Simultaneously, because miR-7 has been characterized as having both oncosgenic and tumor-suppressive properties [65–68], the CDR1as/miR-7 axis is likely involved in cancer initiation and progression [33]. Remarkably, Li and others have shown that circ-ITCH expression is typically downregulated in esophageal squamous cell carcinoma (ESCC) compared to paired adjacent tissue. Circ-ITCH may have an antitumor function in ESCC that acts through interactions with miRNAs such as miR-7, miR-17, and miR-214 and an increase in the level of ITCH, which facilitates ubiquitin-mediated Dvl2 degradation and decreases the expression of the oncogene c-myc. This process therefore inhibits canonical Wnt signaling [30]. Moreover, researchers have found circRNAs are globally reduced in CRC tissues via analyses of RNA-sequencing data from 12 matched normal colon mucosa and tumor tissues [31]. We have also identified that the circRNA expression signatures of PDAC are dysregulated via microarray platform (S.B.Q., unpublished observations). The microarray profile has been deposited in GEO with accession number GSE69362 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69362). These findings indicate that dysregulated circRNAs may be involved in the progression of CRC and PDAC. Finally, circRNAs have also been described as a class of aging biomarkers in Drosophila [32] and as putative disease biomarkers in human saliva [25,33]. Burd and colleagues discovered that circANRIL (circular antisense non-coding RNA in the INK4 locus, circANRIL) is an antisense transcript from the INK4ARF locus. Single nucleotide polymorphisms (SNPs) on chromosome 9p21.3 near the INK4ARF (CDKN2a/b) locus within the atherosclerotic vascular disease (ASVD) risk interval may regulate ANRIL splicing and cANRIL production. Intriguingly, cANRIL expression correlates with INK4ARF transcription and ASVD risk [28]. Moreover, researchers have also discovered that hsa_circ_002059 is downregulated in gastric cancer and could represent a potential novel biomarker for the diagnosis of GC [34]. These findings suggest that circRNAs may be involved in ESCC, CRC, PDAC and GC initiation and progression. As research into circRNAs proliferates, circRNAs may also be found to play roles in other tumors.

Conclusion

CircRNAs were previously largely thought to arise from errors in RNA splicing. However, with the advancements in high-throughput sequencing technologies and bioinformatics progression, the biogenesis and function of circRNAs that have been hidden in the multifarious ncRNAs have drawn the attention of many scientists. More importantly, the study of circRNAs has gradually become one of the most noticeable areas in the field of RNA biology [69]. A circRNA database has been constructed (http://www.circbase.org/) [70]. This database will facilitate further research on circRNAs. In this review, we have described natural circRNAs as an abundant, stable, diverse and conserved class of RNA molecules. Reverse complementary sequences and RBPs play profound roles in circRNA biogenesis, but very little is known about the degradation and localization of most circRNAs. CircRNAs can act as competing endogenous RNAs to bind to miRNAs or regulate transcription or affect parental gene expression, and it seems that other functions will be revealed. Moreover, some circRNAs may be involved in differentiation or disease, especially in cancer. A database of disease-circRNA association in Circ2Traits has also been constructed (http://gyanxet-beta.com/circdb/) [71]. This database enriches knowledge base of potential association of circular RNAs with cancer in humans. CircRNAs are associated with cancer-related miRNAs and some circRNA–miRNA axes may be involved in cancer-related pathways. Hsa_circ_002059 is first found to be significantly downregulated in GC, and may be a potential novel and stable biomarker for the diagnosis of GC [34]. Circ-ITCH expression is typically downregulated in ESCC, and may have an inhibitory effect on ESCC by suppressing the Wnt/β-catenin pathway [30]. Additionally, circRNAs exhibit aberrant expression in CRC [31] and PDAC via high-throughput screening. Although there are just few studies of circRNAs in cancer, studies of circRNAs in cancer are on the way. The prospect of research and applications about circRNAs in cancer is promising. Therefore, we could potentially construct engineered circRNAs as molecular tools or therapies. Engineered circRNAs could be effective either for sequestering miRNAs and other RNAs.
or RBPs or for releasing these stored molecules via cleavage of the circRNA.

Taken together, the functions and related mechanisms of circRNAs may be rather diverse. CircRNAs may affect life processes, serve as diagnostic or predictive biomarkers of disease and also provide new potential therapeutic targets. Nevertheless, compared with coding RNA and miRNA and lncRNA, there are still significant gaps in our current understanding of circRNAs. The circRNA world is full of treasure. CircRNAs have provided new insights into the “dark matter” of the human genome. The latent roles of circRNAs in the diagnosis and treatment of diseases could be massive. Recent advances have primarily focused on the mechanisms of circRNA biogenesis. The biological and molecular mechanisms of circRNAs in the development of diverse diseases are not yet fully understood. With the development of technology and research, additional circRNAs will be identified. Moreover, further studies will reveal the functions of the vast majority of circRNAs in terms of both physiological and pathological processes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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