**Circular RNA: An Emerging Landscape in RNA Biology and Beyond**

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**Abstract**

Circular RNAs (circRNAs) have recently emerged as a fascinating and abundant class of non-coding RNAs, capturing the attention of researchers in the field of biotechnology. These covalently closed RNA molecules were once considered as by-products of splicing errors. However, advances in high-throughput sequencing and bioinformatics have revealed their pervasive presence across various species and tissues. Our chapter intends to summarise the main implication of circRNAs in their biogenesis and degradation mechanisms, their role in altering gene expression, translation into several proteins, as biomarkers for human diseases, their therapeutic potential, and their application in stem cell biology and various computational approaches and tools for their analysis. This chapter aims to encourage the research of circular RNA in the molecular biology field.

**Keywords - Circular RNA, Biogenesis, Biomarker, RNA therapeutics, mi-RNA sponge.**

**I. Introduction**

Circular RNAs are the emerging class of RNA transcripts with numerous applications in various fields such as medicine, agriculture and other allied sectors. They are distinguished from mRNA and other long non-coding RNA molecules by the absence of free 5’ and 3’ ends and polyadenylated tails. They have a covalently closed loop structure arising from back splicing. They are the component of non-coding RNA molecules; still, some are translated into proteins. They play a key role in regulating gene expression at the post-transcriptional level by acting as miRNA and protein decoys. Diener [1], in 1969 discovered the first RNA molecule with a circular structure in potatoes affected with spindle tuber disease. Sanger *et al.* in 1976[2]described viroids that contain single-stranded and covalently closed circular RNA molecules by electron microscopy in the hepatitis delta virus. Later circular transcription of the SRY gene in the testis of mice and through various high throughput sequencing approaches, miRNA sponge function and circular RNA working as competitive endogenous RNA were discovered [3]. Circular RNA is still a research hotspot due to their multiple cellular processes. The simpler circular RNA found in potato spindle tuber is single-stranded RNA about 250-400 nucleotides. In contrast it is about 1.7 kb in case of hepatitis delta virus, a human pathogen. Circular RNAs are more stable and highly resistant to exonuclease degradation due to the absence of free 5’ and 3’ ends. Circular RNAs have a high half-life of about 48h, whereas mRNA is only about 10h. Exons and introns subjected to circularization are three times longer than normal exons and introns, suggesting that length plays a significant role in deciding which exon and intron to circularize [4]. Compared to the mRNA of its host gene, circular RNA has a higher tissue selectivity, suggesting that it has high analytical specificity, robustness, reproducibility and repeatability. They have distinct temporal and spatial expression profiles, and many are evolutionarily conserved due to their stable circular structure [5]. Conservation of circular RNA in mice and humans obtained through tissue-specific enrichment is 92.6%, 3.9%, 2.3%, 1% and 0.1% in the brain, heart, liver, skin and lung, respectively [6]. The highly conserved nature of circular RNA made them a biomarker for various human diseases, mainly cancer. The high expression of more than 10 000 circular RNA in the brain suggests that they have a role in healthy brain function, particularly learning and memory, due to their localisation in synapses [7]. It is found that the timely expression of circular RNA in muscle plays an essential role in the growth regulation of mammals so that it can act as growth marker. The homeostatic expression of circular RNA gets altered due to the onset and development of various pathologies and diseased conditions. Understanding the mechanism behind circular RNA will lead to the development of different diagnosis and prognosis tools and targeted therapies for multiple diseases.

**II. Biogenesis of circular RNA**

The mechanism of formation of various types of circular RNA is highly diverse. pre-mRNA, pre-tRNA, and pre-rRNA are the primary sources for forming circular RNA [8]. Lariat formation and direct back splicing are the two main mechanisms through which pre-mRNA can be spliced into circular RNA [9]. Lariat-derived circRNA formation can be achieved through exon skipping and direct circularisation of the intronic lariat. A hetero-lariat structure formation is there in case of exon skipping resulting in the formation of exon-intronic circular RNA (EIcircRNA), which sometimes continue with the removal of all introns and leads to the origination of exonic circular RNA (ecircRNA) [10]. Direct circularisation of intronic lariat is by pre-mRNA cleavage at the 5’ end with the help of small nuclear RNA (U1snRNA) and ligated between 5’ guanidine and 2’ adenosine. The formed intronic circular RNA is retained inside the nucleus [11]. Back splicing can generate all three types of circular RNA; exonic circular RNA, exon intronic circular RNA, and intronic circular RNA [12]. Back splicing follows RNA binding protein-driven and intron-pairing-driven circularisation. Two flanking introns can be bridged through RNA binding proteins, which form a circularised structure and a linear product [13]. The direct base pairing of Alu elements happens in intron-pairing driven circularisation, which facilitates the ligation of the 5’ donor and 3’ splice acceptor site and forms a circularised structure and a linear product. The introns are removed or retained to create an ecircRNA or an EIciRNA (exon-intron circRNA) [14]. tricRNA called tRNA-driven intronic circular RNA [15] and rRNA - derived circular RNA [16], follows the same process which is reported in bacteria and to some extent in eukaryotes [17]. A detailed explanation of biogenesis is given in Figure 1.

**III. Regulatory mechanisms of circular RNA**

Circular RNAs can regulate various cell functions due to their high stability and prolonged half-life. Different regulatory mechanisms of circular RNAs are listed below.

**a) Circular RNA- Gene expression regulators**

CircRNAs can control the expression of parental genes in various ways, including microRNA (miRNA) sponges, mRNA traps and control of transcription and splicing. Circular RNAs are primarily formed in the nucleus and exported to the cytoplasm by various exportin proteins such as UAP56 and URH49, but the export mechanism is still unclear. Nevertheless, several circular RNAs remain in the nucleus and regulate the pol II-mediated transcription. They usually regulate the transcription at initiation, elongation and post-transcriptional levels. The pre-initiation complex formed in the promoter of eukaryotic transcript is further stabilised by various transcription factors, which stimulate the transcription rate. To find whether other factors or non-coding RNAs are involved in promoting transcription, Li et al. in 2015 [18] performed an immunoprecipitation assay using RNA polymerase II specific antibody, where they found about 111 circular RNAs are involved in promoting pol II transcription. Of these 111 circular RNAs, 15 of them are EIcircRNA. Fluorescent *In situ* Hybridization (FISH) proved that two among them are circEIF3J and circPAIP2, which are exclusively localised in the nucleus. Knockdown of these two resulted in decreased transcripts produced, proving that binding circular RNA in the initiation complex promotes the transcription initiation level. It is almost believed that introns are an unused part of transcription, but it has been

found that several intron-derived circular RNAs are involved in regulating transcription elongation [19]. ci-ankrd52 is formed from the second intron of the ankrd52 gene, which is engaged in promoting transcription elongation, synthetic antisense oligonucleotides, which is complementary to the ci-ankrd52 which, when used, will reduce the number of mRNA produced, which proves that intronic circular RNAs are engaged in transcription elongation [20]. The primary function of circular RNA found to date is its ability to act as a mi-RNA sponge. Almost about 18,000 microRNA have been found to date. These mi-RNAs will downregulate the gene expression by binding to the mRNA. Circular RNA promotes the expression of mRNA by binding to those inhibitory mi-RNAs. ciRS- 7, generated from the antisense transcript of CDR1as, is the first reported circRNA. It acts as a mi-RNA sponge and negatively regulates miR-7 by expressing in brain tissue, neuroblastoma and astrocytoma. More than 60 binding sites for miR-7 are found in CDR1as, and its resistance to miRNA-mediated degradation makes it the perfect ceRNA for miR-7 [21]. Apart from CDR1as, several other circular RNA such as cir-SRY, cir-ITCH, circ-HIPK3, circ-PVT1, circRNA-CER, circRNA- MYLK etc. are involved in miRNA sponging function [22]. The circRNA may act as an mRNA trap by securing the start site, producing a noncoding linear transcript, and reducing the expression level of the beneficial protein when the host gene's translation start site is present. Duchenne muscular dystrophy (DMD) sufferers have also been discovered to have the mRNA trap phenomena. According to a study by Gualandi *et al.* 2003 [23], in people with specific deletion mutations, greater dystrophin EcircRNA production may result in inactive dystrophin transcripts and lower quantities of functional proteins [24].

**b)** **Circular RNA in Protein translation**

Circular RNA, which falls under the long non-coding RNA, sometimes gets translated into proteins. The presence of open reading frames, internal ribosome entry sites that acts as RNA scaffold interacting with translational machinery, and N6-methyladenosine modifications that interact with YTH domain family protein 3 (YTHD3) and recruit eIF4G2 to start the translation indicate that the circular RNA undergoes cap-independent translation [25] [26]. Proteins produced from circRNA are involved in myoblast proliferation (circZNF609 - 250 AA) [27], inhibit transcription elongation (circPINTexon2 in glioblastoma cells – 87aa) [28], inhibits ubiquitination of β-catenin and activates Wnt/β-catenin signalling (circβ-catenin - 370 AA) [29].

**c) Circular RNA – protein interactions**

Even though most circRNAs do not contain multiple protein-binding sites, many circRNAs can act effectively as protein sponges. Circ-Amotl1 binds to PDK1 and AKT1 through binding sites to mediate wound repair [30]. CircRNAs function as protein decoys and cooperate with the target protein at the appropriate cellular site to change its routine physiological function. For example, circ-Amotl1 can bind and retain c-Myc in the nucleus, where it stabilises c-Myc, up-regulates its target genes, and leads to increased cell proliferation, reduced apoptosis, and a highly tumorigenic phenotype [31]. CircRNAs can also function as scaffolds facilitating contact between two or more proteins. Some circRNAs, such as circ-Amotl1 and circ-Foxo3, function as protein scaffolds to facilitate the co-localization of enzymes and their substrates [30]. CircRNAs may also recruit specific proteins to certain cellular locations, as exemplified by the circRNA, FECR1, which recruits Ten-Eleven Translocation methylcytosine dioxygenase 1 (TET1) to the promoter region of FLI1, its host gene, leading to the demethylation of CpG sites and active transcription [32]. Currently, the interactions between circRNAs and proteins are mainly analysed by RNA pull-down assays or RNA immunoprecipitation (RIP); genome-wide profiling, locus-specific profiling, and visualisation of circRNAs are also used to discover, profile, and understand the biogenesis and functions of circRNAs [33].

**d) Circular RNA as biomarkers for human disease**

Due to its high stability and vast utility, Circular RNA can be used as biomarkers for various diseases, particularly cancer. Circular RNA is the primary key player in almost all types of cancer as a biomarker. Apart from cancer, it can be used as a diagnostic biomarker in other pathologies such as hypertension, cardiac diseases, diabetes, brain disorders etc. also it can be used as a prognostic biomarker for several ageing and age-related disorders. In addition to these disorders, circular RNAs are involved in several gynaecological and reproduction-related disorders regulating follicular maturity. Table 1 discusses the circRNAs that serve as biomarkers in various diseases and their targets.

**IV. Degradation pathways of circular RNA**

Due to the absence of free 5’ and 3’ ends, endo-nucleolytic cleavage is the only possible way of circular RNA degradation. RNase L, which is circRNA endonuclease involved in globally degrading circular RNA [51]. Kim et al.'s [52] discovery that HRSP12, which RNase P/MRP particularly downregulates, is required for the relationship between a subset of circRNAs containing m6A and YTHDF2 is supported by this. Finding that m6A can mediate both circRNAs and mRNA degradation appears to have raised awareness of this circRNA degradation mechanism among researchers [53]. In several situations, binding of mi-RNA to circular RNA leads to

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| --- | --- | --- | --- | --- |
| **Disease**  | **circRNA** | **Target** | **Effect on the disease** | **References** |
| Ovarian cancer | circ\_0072995 (Upregulated) | miR-147a | Promotes cell proliferation and invasion and suppresses apoptosis | [34] |
| circ\_MTO1(Downregulated) | miR-10a | Suppresses cell proliferation and promotes apoptosis |
| Gastric cancer | circ\_0000936(Upregulated) | miR-582-3p/ HUR/VEGF | Promotes cell proliferation, migration, invasion and angiogenesis | [35] |
| Breast cancer  | circRNA\_002178(Upregulated) | miR-328-3p/COL1A1 | Promotes cell proliferation, invasion, angiogenesis, and inflammatory response and decreases overall survival. | [36] |
| circ\_0000515(Upregulated) | miR-269-5p/CXCL10 |
| Hepatocellular carcinoma | circRNA-100338(Upregulated)  | MMP9/ MVD/ VE-Cadherin /ZO-1 | Promotes invasion, metastasis and angiogenesis | [37] |
| Renal Cell Cancer | circPRRC2A(Upregulated) | TRPM-3 | Promotes angiogenesis and metastasis | [38] |
| Bladder cancer | circSEMA5A(Upregulated) | miR-330-5p/ ENO1/ SEMA5A | Promotes cell proliferation, suppresses apoptosis, facilitates migration, accelerated invasion, enhanced angiogenesis, and promotes glycolysis | [39] |
| Prostate Cancer | circRNA\_001587(Downregulated) | miR-223/SLC4A4 | Inhibits migration, invasion and angiogenesis | [40] |
| Osteosarcoma | circTADA2A(Upregulated) | miR-203a-3p/ CREB3 | Promotes metastasis and inhibits apoptosis | [41] |
| Hypertension | Hsa\_circ\_0005870(Upregulated) | hsa-miR-6807-3p, hsa-miR-5095, hsa-miR-1273g-3p, hsa-miR-5096, hsa-miR-619-5p | - | [42] |
| Cardiovascular disease | circHRCR(Downregulated) | miR-223 | Inhibits cardiac hypertrophy and heart failure | [43] |
| circHECTD1(Upregulated) | miR-142-TIPAR | Astrocyte activation and cerebral infarction in stroke | [44] |
| circMFACR(Upregulated) | miR-MTP18 | Cardio myocyte death in myocardial infarction | [45] |
| CircFOXO3(Upregulated) | ID-1, E2F1, FAK, H1F1α (Transcription factors) | Promotes cardiac senescence by arresting ID-1, E2F1, FAK, and HIF1a in the cytoplasm | [46] |
| circANRIL(Downregulated) | PES1 | Controls ribosome biogenesis in atherosclerosis | [47] |
| Alzheimer's disease | ciRS-7(Downregulated)  | miR-7-UBE2A | Modulates the α-synuclein aggregation pattern | [48] |
| Parkinson’s disease  | ciRS-7(Upregulated) | miR-7-PD |
| Diabetic retinopathy | circ\_0005015 (Upregulated) | miR-519d-3p | Facilitates retinal endothelial angiogenic function  | [49] |
| circHIPK3(Upregulated) | miR-30a | Regulates retinal endothelial cell function and vascular dysfunction  |
| Osteoporosis | circRUNX2 and circ\_0002060 (Downregulated) | miR-203 | Promotes the expression of osteogenic differentiation-related proteins | [50] |

**Table 1. Circular RNAs as disease biomarkers and its respective targets.**

endonucleolytic cleavage of circular RNA and finally subjects to complete degradation. When miR-671 binds to CDR1as, the endonuclease Argonaute-2 (Ago2) directly cleaves the RNA, producing a 5' and 3' free end. The broken-down RNA is destroyed by various exonucleases on both ends [54]. Another study found that the depletion of GW-182 in DL1/S2 cells in Drosophila increased the amount of circular RNA transcripts, proving that the gawky 182 protein is responsible for circular RNA degradation [55]. Further research confirmed that RNA binding proteins UPF-1 and G3BP1 are involved in the structure-mediated degradation of circular RNAs. Similar to the gawky-mediated degradation, the depletion of UPF-1 and G3BP1 promotes the upregulation of circular RNA, evidencing that those RBPs are responsible for the degradation of circular RNAs [56].



**Figure 1. Biogenesis and degradation mechanisms of circular RNA. IRES: Internal Ribosome Entry Site; ecircRNA: exonic circular RNA; EIcircRNA: Exon-intron circular RNA; ciRNA: Circular intronic RNA.**

**V. CircRNAs in Development and Physiology**

Circular RNAs are expressed in several organs following a spatial- and temporal-specific course, suggesting their potential bio functions [57]. To date, there is a growing number of studies reporting that circRNAs could be involved in the development of mammalian tissues as in neural development [58], osteogenic differentiation [59], skeletal muscle development [60] or in hematopoiesis [61]. CircRNAs play a pivotal role in promoting the myogenesis process in the skeletal muscle by acting as miRNA sponges inhibiting myoblast differentiation. E.g., circ-zfp-609 promotes myogenesis by sponging miR-194-5p and upregulating BCLAF1 [62]. CircRNAs, namely circBIRC6, maintain the pluripotency in human embryonic stem cells by acting as miRNA (miR-34a and miR-145) sponge, relieving the suppression of NANOG, OCT4 and SOX2 expression [63]. Numerous studies have shown that circRNAs play an essential role in the osteogenic differentiation of Bone Marrow Stem Cells (BMSCs), collagen synthesis, and FGF or TGF-β signalling pathways in the commitment towards chondrogenic differentiation [64].

**VI. Therapeutic Potential of circRNAs**

The circRNAs dysregulation affects cancer cell proliferation, invasion, migration, angiogenesis, apoptosis, or drug resistance, thereby affecting the progression of a diversity of cancer. The advantage of targeting circRNA treatment lies in its potential for low off-target effects. In contrast, microRNAs and small interference RNAs have higher off-target effects owing to their short length and half-life [65]. CircRNAs represent an emerging, robust mechanism for delivering therapeutics and vaccines due to their closed-loop structure resistant to nuclease degradation, protein-coding potential, and improved stability compared to their linear mRNA counterparts. Artificial circular RNA (circRNA) sponges (circmiRs) are potentially effective therapeutic miRNA antagonists [66]. The successful development and application of COVID-19 mRNA vaccines have prompted researchers to explore using circRNAs to develop higher-stability nucleic acid-based COVID-19 vaccines. Low doses of circRNA vaccines may be sufficient to elicit strong immune responses [67]. Researchers have also attempted to utilise the stability of circRNAs for the direct synthesis of chimeric antigen receptor (CAR) T cells by introducing CAR-expressing circRNA into T cells to avoid the tedious process of producing CAR-T cells through T cell isolation followed by engineering [68]. The use of nanoparticles as a delivery mechanism has greatly increased the in vivo feasibility of circRNA-based therapies [69].

**VII. Circular RNA and RNA Editing**

Several strategies of RNA editing are used to study circRNA. Circular RNAs are cleaved by delivering siRNA/shRNA lipid-based polymers in vivo by targeting their back-splice junction. This RNAi-mediated knockdown of circular RNA has various disadvantages, including rapid nuclease degradation, limited intracellular transport efficiency, absence of cell-specificity, the immune response, and other off-target consequences [70]. Conditional circRNA knockout/knockdown is utilised to trigger circRNA cleavage in certain cells in vivo using a cre-dependent short hairpin RNA (shRNA) which is then processed into short interfering RNA (siRNA) [68]. Lipid nanoparticles (LNPs) mediated siRNA delivery system targets circRNAs in the cytoplasm of specific cells using endogenous or exogenous ligands. Since nanoparticles cannot penetrate the nucleus, this method is confined to targeting circRNAs in the cytoplasm [69]. Lentiviral and adenoviral circRNA expression plasmids are used to overexpress circRNA vectors in vivo [71]. Circular RNA knockout using CRISPR/Cas9 removes the intronic complementary region flanking the circularised exon important in circRNA synthesis. This technique has also been used to knockout and knockdown targeting the entire gene locus and a transcription factor, respectively. In this case, it is difficult to determine the intron to be targeted due to the complex circRNA biogenesis. This challenge can be subdued by CRISPR/Cas13-mediated circRNA knockdown, which targets the back-splice junction of circRNAs rather than introns to induce circRNA cleavage with less off-target consequences [72].

**VIII. Computational Approaches for Circular RNA Analysis**

The advent of RNA sequencing has enabled researchers to profile the transcriptome for circRNAs. Several bioinformatics tools have played a pivotal role in deciphering the functional significance of circular RNA using RNA seq data or even transcript sequence features. There are various methods for circRNA library preparation, such as polyA selection, polyA depletion, rRNA depletion, RNase R treatment, RNase R treatment, followed by polyadenylation and polyA (+) RNA depletion. Based on the implementation, circRNA identification tools can be divided into three categories – Back splicing junction (BSJ)-based, integration-based, and machine learning-based. The algorithms used for circRNAs identification by BSJ are further classified into split-alignment-based (Segmented based) and pseudo-reference-based (Candidate based). Segmented-based tools identify BSJ by aligning reads to the reference genome and are considered de novo tools. In contrast, pseudo-reference tools rely on gene annotation files to identify junction reads [73]. Some tools follow integration pipelines for identifying circular RNA with high reliability. Standard features of circRNAs, such as ALU repeats, structure motifs and sequence motifs, are used for building reliable classification models in machine-learning techniques for circRNA identification. Some circRNA identification tools also include the ability to quantify the expression levels of circRNA. Many circRNA databases collect putative circRNAs from the literature based on various identification tools and specific NGS datasets. Certain databases collect all ncRNA information with circRNA as a part, circRNAs associated with diseases, circRNA interaction with miRNA, RNA binding proteins and Transcription factors (TFs). Various tools for downstream analyses of circRNA are capable of alternative splicing detection, circRNA assembly, primer design, structure prediction, and visualisation. The computational tools of circRNA and their applications with bioinformatics workflow are depicted in Fig.2. Despite numerous carefully curated circRNA databases, there is a lack of standardised nomenclature for circRNA annotation, leading to non-universal IDs used across these databases. This poses a significant challenge in the field of circRNA research



**Figure 2. Bioinformatics pipeline for circRNA identification and analysis with the** **list of computational tools used in each step**

**IX. Conclusion**

 Researchers were already beginning to uncover various functional roles of circRNAs, including their involvement in gene regulation, RNA-binding protein interactions, and potential roles in disease development. As circular RNA (circRNA) research advances, several challenges must be addressed. Accurate detection and quantification of circRNAs can be challenging due to their low abundance compared to linear RNAs, which require more tools that are reliable. Determining the specific functions of circRNAs remains a complex task. Comprehensive studies of circRNA across different cell types and tissues remain unexplored as they exhibit tissue-specific expression patterns. In the future, we require a deeper understanding of the specific functions of circRNAs and how they contribute to cellular processes. Identifying and linking circRNAs to specific diseases could open new avenues for diagnostics, targeted therapies and biomarker development for prognostics and disease monitoring. CircRNAs' tissue-specific expression patterns and disease associations could contribute to personalised medicine approaches, enabling tailored treatments based on individual patient profiles. Integration of circRNA data with omics data is necessary to provide a holistic view of cellular processes and biological mechanisms. Considering the stability and cytoplasmic localisation of circRNAs, engineered circRNAs could be used for various molecular tools or therapies**.** As circRNA research matures, it will likely intersect with other areas of biology, genomics, and medicine, leading to exciting discoveries and applications. However, researchers must address the challenges associated with circRNA research to fully unlock their potential and shed light on their roles in health and disease.

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