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REVIEW

Comprehensive analysis of circular RNAs in pathological states: biogenesis, cellular regulation, and therapeutic relevance

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Abstract

Circular RNAs (circRNAs) are members of the non-coding transcriptome; however, some of them are translated into proteins. These transcripts have important roles in both physiological and pathological mechanisms due to their ability to directly influence cellular signaling pathways. Specifically, circRNAs are regulators of transcription, translation, protein interaction, and signal transduction. An increased knowledge within their area is observed over the last few years, concomitant with the development of next-generation sequencing techniques. circRNAs are mostly tissue and disease specific with the ability of specifically changing the biological behavior of cells. The altered expression profile is currently investigated as novel minimally invasive diagnosis/prognosis tool and also therapeutic target in human disease. The diagnosis approach is based on their level modification within pathological states, especially cancer, where circRNAs' therapies are intensively explored in anti-aging strategies, diabetes, cardiovascular diseases, and malignant pathologies, and are relying on the restoration of homeostatic profiles.

Keywords Circular RNA · Biogenesis · Gene expression regulation · Human disease · Biomarkers · Databases · Therapy

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Abbreviations

Ago2	Argonaute 2
circRNAs	Circular RNAs
EGFR	Epidermal growth factor receptor
ElcircRNA	Exon–intron circular RNA
EMT	Epithelial-to-mesenchymal transition
fcircRNA	Fusogenic circRNA
lncRNAs	Long non-coding RNAs
m^6A	N^6-Methyladenosine
miRNAs	MicroRNAs
ncRNAs	Non-coding RNAs
siRNAs	Small interfering RNAs
tricRNAs	tRNA intronic circular RNAs

Introduction

Since their discovery, significant improvements have been made in confirming the consequential role of non-coding RNAs (ncRNAs) in the coordination of physiological and pathological processes [1–7]. Nowadays, the transcription of the human genome into ncRNAs is well characterized. The major types of ncRNAs are: microRNAs (miRNAs), small interfering RNAs (siRNAs) and long non-coding RNAs (lncRNAs) [4, 5]. Intensive efforts are made to study the

role of ncRNAs, with a special focus on their interactions with various transcripts and proteins [3, 8].

Recently, the previous status of some ncRNAs called circular RNAs (circRNAs) has been revised [9], being highlighted new functional roles [4, 10]. These transcripts have multiple cellular functions and the alteration of their homeostatic expression is associated with the onset and development of various pathologies [3, 11, 12]. Deep understanding of these mechanisms will impact a wide range of clinical niches, particularly within the development of new diagnosis and prognosis tools and targeted therapies [4, 10].

The history of cirRNAs began around 45 years ago, with an article published by Sanger et al. which described the sequencing method for bacteriophage F1 genome, focused on the analysis of nucleotide polymers found in a close loop [13]. Later on, with the help of electron microscopy and electrophoresis in the hepatitis delta (δ) virus, circRNAs were discovered [14]. In 1991, it was assessed that the exons of the tumor suppressor gene *DCC* (deleted in colorectal cancer) are joined through their splice site in a different order [15]. Two years later, circRNAs containing exons were found in the cytoplasm of eukaryotic cells [16]. At that time, they were considered a result of defective splicing [17]. However, circRNAs are highly conserved sequences due to their stable circular shape [18] and after general reconsideration regarding the role of ncRNAs in organism homeostasis and progress to a pathological state, circRNAs' previous status has been revised [19–21].

Our paper intends to summarize the main implication of circRNAs in cellular communication and the translation of these mechanisms in pathological scenarios. Specifically, we present the mechanism of circRNAs biogenesis, their roles in cellular processes and the various methods used for their detection and quantification, together with the databases developed for their analysis in human disease. The general overview of circRNAs intends to encourage the progression of clinical uses of these molecules as biomarker or therapeutic tools.

Circular RNAs: definition and biogenesis

Nowadays, circRNAs are considered an important bridge between the coding and the non-coding RNAs. Initially, circRNAs were thought to be only the effect of the transcriptional noise [17], but further studies challenged this idea [19, 21, 22] through the identification of thousands of genomic loci in mammals specific for circRNAs; moreover, these sequences have important implication in cellular state [23].

CircRNAs are generated from intronic or exonic sequences [24] found in thousands of genomic loci [23]. Some of them are transcribed from a single gene or multiple genes [25]. An example from multiple coding genes is the *PML/RARα* fusion gene in leukemias. The primary transcript can undergo the process of back-splicing and give rise to a fusogenic circRNA (fcircRNA) that contains exons from both translocated genes [26].

CircRNAs can be originated from the primary form of: messenger RNA (pre-mRNA), ribosomal RNA (pre-rRNA), or transport RNA (pre-tRNA) [27] (Fig. 1). The pre-mRNA can be spliced into circRNAs through two main mechanisms: lariat formation and direct back-splicing [28, 29].

Lariat formation mechanism is divided into two types of strategies: direct circularization of intronic lariat and circularization through exon skipping [17]. For the direct circularization of an intronic lariat, the pre-mRNA is cleaved at the 5′ end with the help of small nuclear RNA (snRNA) U1 and ligated through a 5′–2′ bound between a guanidine and an adenosine [30]. The intronic lariat is processed and kept in a circular form in the nucleus [31]. In the case of exon skipping, a "hetero-lariat" is formed, containing both exons and introns (ElcircRNA) [32, 33]. This strategy can continue with the removal of all introns and the generation of circular RNAs containing only exons, which are named exonic circular RNAs (EcircRNAs) [28].

Back-splicing can generate all of the three types of circular RNA: circular intronic RNAs (ciRNAs), EcircRNAs, and intron–exon circular RNAs (EIcircRNAs) [22]. Through the process of direct back-splicing, the 5′ splice donor is ligated to a 3′ splice acceptor via simple or RNA-binding proteins (RNP) base-pairing [15, 34]. This is facilitated by the presence of Alu repeats in the intronic area [28, 35]. There are various *cis*- or *trans*-acting factors responsible for bringing the donor and acceptor sites in proximity to one another [20]. Although the canonical splicing shares common regulatory factors with the alternative splicing, the interaction pattern discriminates between the two [36]. Moreover, some of these factors, such as nuclear factor 110 (NF110) [37] or the Quaking (QKI) protein [38, 39], remain bound to the circRNAs also in the cytoplasm and enhance their stability.

A greater diversity of circRNAs is found in bacteria (derived from primary transcripts of tRNAs [40] or rRNAs contain introns [41]). The introns of pre-tRNA are cleaved and circularized. These RNA products are named tRNA intronic circular RNAs (tricRNAs) and were discovered both in bacteria and in eukaryotic cells [40, 41]. In bacteria, the intron containing pre-rRNAs is passing through a similar process, resulting in a circularized rRNA transcript [41].

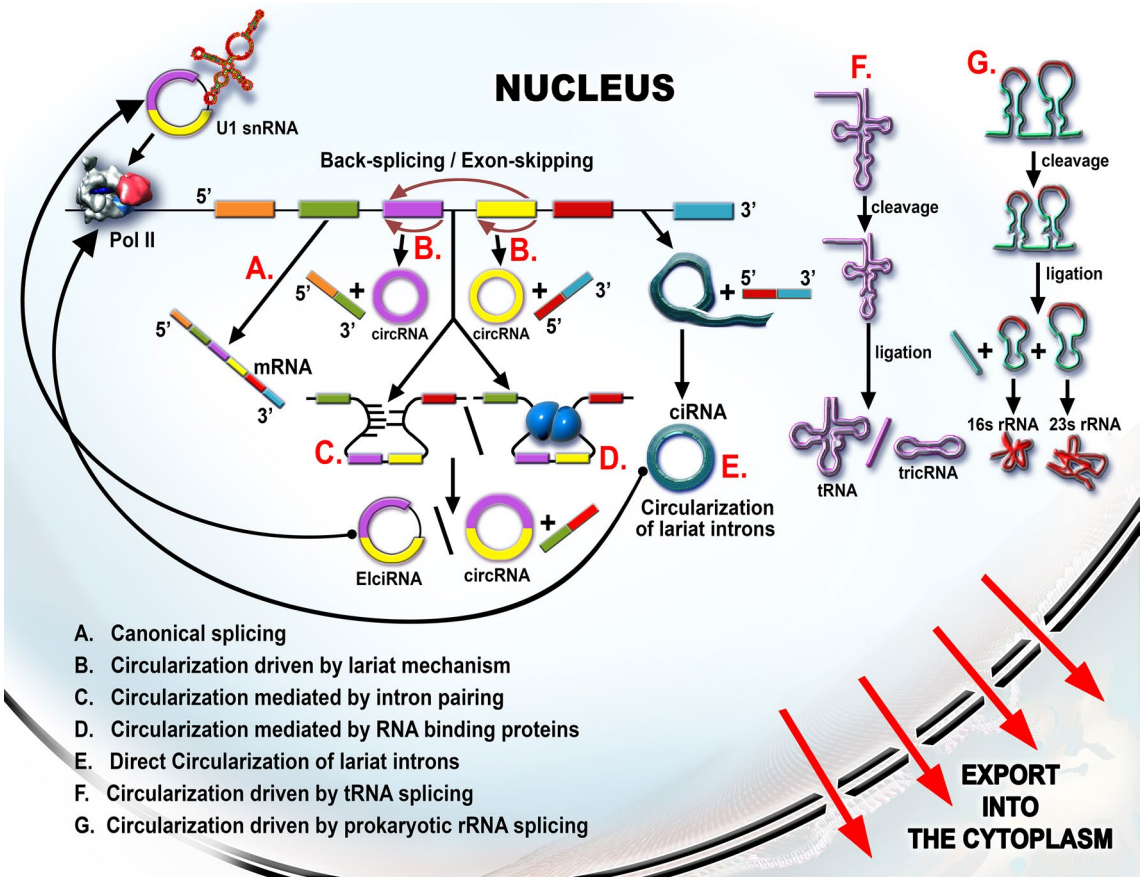

Fig. 1 The biogenesis of circRNAs. **a** Through the pre-mRNA canonical splicing, the mature mRNA is generated. The alternative splicing pathways are: exon skipping and back-splicing. **b** The circularized transcript can contain only the exon part and it is named exonic circular RNA (ecircRNA/circRNA). **c**, **d** The flanking introns can form direct base-pairing (**c**) or RNA-binding protein (RNP)-mediated intron–intron binding (**d**). The resulted circular transcript can be an exon–intron circular RNA (EIcircRNA) or the introns are further removed and the two- or multiple exons containing ecircRNA are generated. The EIcircRNA can regulate its own transcription by interacting with U1 snRNA and RNA Pol II. **e** The intron can form a lariat during canonical or alternative splicing. The lariat may become more stable and circular thus giving rise to the intronic circular RNA (circRNA). **f** In bacterial cells, as well as in eukaryotic cells, it was proven that the primary tRNA has introns that are spliced and circularized into tricRNA. Their function still remains to be studied

CircRNAs' biological function

Important roles are now attributed to circRNAs sequences, probably with many others to be discovered. These transcripts are key regulator molecules, being also highly conserved due to their lack of 3′ poly-A tail, 5′ cap, and consequential circular shape [18]. circRNAs fulfill a variety of functions inside the cell [42] through their interaction with DNA, other coding or non-coding RNAs, and proteins [27].

CircRNAs are involved in the regulation of transcription, post-transcription control (particularly splicing), and translation and post-translational signaling [17, 43]. They act as competing endogenous RNAs (ceRNA) for miRNAs [44, 45], with important consequences in the regulation of gene expression. It is supposed that their miRNA sponging activity is also connected with lncRNAs function; however further experimental data are needed [46]. CircRNAs functions are summarized in Fig. 2.

CircRNAs as gene expression regulators

CircRNAs are generally tissue-specific [24], where 20% of the expressed genes are able to generate circular transcripts [47]; therefore, they are correlated with the expression profile of the parent genes [19]. As stated, circRNAs are involved in transcription and post-transcriptional gene expression regulation, including interference with key RNA-binding proteins.

CircRNAs and transcriptional control

circRNAs are active elements at transcriptional level, acting as positive regulators of Pol II transcription through their

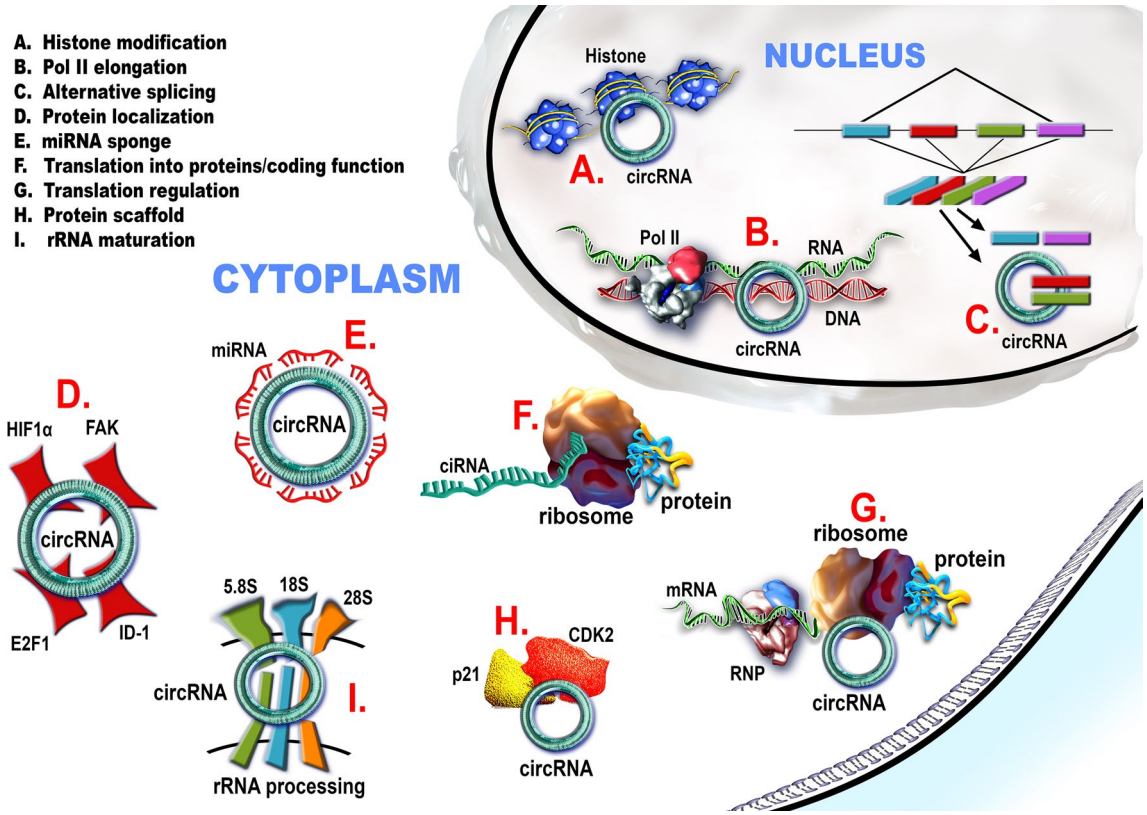

Fig. 2 Circular RNAs (noted circRNA—for all circular RNA types) fulfill multiple functions inside the cell. In the nucleus **a**–**c** circRNAs can **a** interact with the histone methylation pattern and silence a specific locus; **b** regulate the transcription of their gene of origin through direct (circRNAs) or snU1-mediated (EIcircRNA) interaction with the RNA polymerase II; **c** compete with the mRNA for the available splicing machinery and interfere in the alternative splicing process. In the cytoplasm **d**–**i** the circRNAs can: **d** entrap certain transcription factors in the cytoplasm; **e** act as miRNAs sponges, especially the circRNAs with exon-containing transcripts; **f** be translated into proteins. **g** circRNAs interact with RNA-binding proteins and regulate the translation of some mRNAs. **h** circRNAs can act as protein scaffolds. They can entrap some proteins and impair their signal transduction role. **i** circRNAs can induce apoptosis by interfering within the processing of pre-rRNA subunits

accumulation at the transcription site [48]. The circRNA ci-ankrd52 is a *cis*-acting positive regulator of its own gene, ANKRD52, by affecting the Pol II-mediated elongation during transcription [49]. The exonic–intronic circRNAs also called EIciRNAs are proved to increase the expression level of the parental gene through a transcriptional control that implies the interaction with the parental genes as *cis* forms, highlighting an important transcription regulatory strategy [50]. These transcripts can also interfere with the histone methylation pattern: e.g., circANRIL interaction with the polycomb repressive complex 1 is necessary for an effective histone 3-methylation and silencing of INK4a locus [51]. Circ-Amotl1 promotes the nuclear localization of the c-Myc transcription factor [52].

The EIciRNAs, circEIF3J and circPAIP2, stimulate the transcription of their own genes by interacting with the transcriptions machinery. These EIciRNAs form a direct RNA–RNA interaction with the U1 snRNA. TheE-IciRNA–U1snRNA complex interacts with the U1A and U1C proteins, U2 snRNA and RNA polymerase II. The EIciRNAs are *cis*-regulators of their gene of origin, but they can also act as *trans*-regulators of distant genes [50]. This is the case of ciANKRD52 that interacts with the transcription elongation factor Pol II [49]. CircRNAs transcripts can also interact with regulatory proteins, affecting the translation of targeted mRNAs. For instance, CircPABPN1 inhibits the HuR protein, a positive regulator of PABPN1 mRNA, thus downregulating the translation of its own gene of origin [53].

CircRNAs and post-transcription control

Alternative pre-mRNA splicing is a contributor of post-transcriptional regulation and sustains the proteomic diversity [46]; circRNAs interfere with this mechanism. In the case of circMbl, the introns contain putative-binding sites for the MBL protein; thus, the MBL gene creates a negative-feedback loop to regulate its own expression mediated by splicing competitiveness [54]. The RNA-binding proteins (RBPs)

act as an ON/OFF switch for lariat formation/back-splicing by facilitating or hindering the complementary base-pairing of the flanking introns [55]. The EcircRNAs entrap the exons from pre-mRNA and disrupts their translation [56]. There are also RBPs, such as ADAR1, which oppose the interaction between the two flanking introns by melting the newly formed stems of dsRNA and impairing the circRNA formation [57]. These introns usually contain reverse complementary sequences, such as AG–CT or GGG–CCC repeats, which allow the interaction with each other [55].

CircRNAs are able to impair rRNA processing: circANRIL induce apoptosis by inhibiting the rRNA nuclease processing and ribosome biogenesis [58].

In general, circRNAs are derived from exonic regions of pre-mRNAs, the fact that anticipates that their synthesis is implicitly disrupting the splicing of the mRNA sequences [56]. The preference toward back-splicing/exon skipping and circularization versus canonical splicing is regulated by the introns flanking the skipped exons. These are long introns, difficult to cut during splicing, and contain reverse complement sequences, which allow the interaction with each other [48].

CircRNA as miRNA sponging

The initial circularized transcript which contains both introns and exons is further processed into a final transcript in which the exon is either kept or discarded. If, in the final circularized form, the exon is present, the circRNAs have the ability of miRNA sponging, as in the case of ciRS-7 from *Mbl* gene and miR-7 [59]. This mechanism is mediated by the binding to the miRNA-specific RBP, Argonaute 2 (Ago2) [46, 60, 61]. The sponging capacity has important clinical applications in tumoral pathologies for targeting oncogenic miRNAs.

CircRNAs can be translated into proteins

Until recently, there were a lot of controversies regarding the translation of circRNAs. Part of them has the necessary characteristics to be translated into proteins, but the actual proteins were only recently found [62]. Some circRNAs have the full set of characteristics to be translated in proteins, which renders them as protein-coding RNAs. N^6-Methyladenosine (m^6A), is the most frequent modification of RNA and this alteration sustains protein translation from circRNAs [25, 63]. Moreover, these circRNAs interact with the ribosome [25] through their internal ribosome entry sites (IRESs) located in the 5′ untranslated region (5′ UTR). These circRNAs possess start and stop codons, similar to their corresponding linear mRNA [64]. With the help of mass spectrometry PRIDE database, 46 translated circRNAs were found, originated in 37 genes [65]. circMbl encodes a protein found in Drosophila [38]; circ-ZNF609 controls myoblast proliferation and is translated into a protein [64]; circ-SHPRH encodes the SHPRH-146aa protein, which functions as a tumor suppressor in human glioblastoma [66]. The bioinformatics tools demonstrate clearly that some circRNAs can be translated, but the probability that circRNAs are endogenously translated has been only indirectly tested [25]. In addition, the protein-coding capacity in the majority of circRNAs in vivo is still a matter of debate.

CircRNAs can entrap proteins and impair signal transduction

CircRNAs act as scaffold for some transcription factor at cytosolic level or nuclear level, as regulatory mechanisms or as transport systems [62]. CircRNAs perform vital functions in regulating a variety of signaling pathways like mitogen-activated protein kinase (MAPK), cell division kinases, phosphatidylinositol 3-kinase (PI3K), or Wnt/β-catenin pathway [67]. CircITCH is an important regulator of cell cycle and, at the same time, affects malignant transformation via Wnt signaling pathway [68].

CDK2 helps in the progression of cell cycle, while the p21 protein opposes its action. The circRNA fork head transcription factor, circ-Foxo3, entraps CDK2 and p21, and hence, it impairs cell cycle progression [69]. In the cytoplasm, circ-Foxo3 is able to bind the stress-activated transcription regulators: ID1, E2F1, FAK, and HIF1α, and block their nuclear import [70]. Therefore, circ-Foxo3 does not affect the expression level of these transcription factors, but it affects their localization. The ID1 [71], E2F1 [72], FAk [73], and HIF1α [74] are cancer-promoting transcription factors, which may explain why the circFoxo3 is underexpressed in cancer [45].

Foxo3 protein and circ-Foxo3 function as positively regulated tumor suppressors. In this case, the circ-Foxo3 has two functions: one is the binding, ubiquitination, and induced degradation of p53, and the other one consists in the interaction with MDM2 protein, thus hindering the MDM2 capability of ubiquitination and degradation of Foxo3 protein [25, 45, 75]. The exact mechanism by which circ-Foxo3 is capable of binding and entrapping these proteins needs to be elucidated [69].

Another important mechanism of gene expression regulation mediated by circRNAs consists in the HuR-mediated translational repression. The CircPABPN1 (hsa_circ_0031288) competes with PABPN1 mRNA for the binding of HuR protein. The HuR protein is a RNA-binding protein (RBP), indispensable for the ribosomal translation of the mRNA; thus, by entrapping the HuR protein, the circRNA suppresses the expression of the linear form. As the level of circRNAs increases, the level of available HuR is decreased [53].

CircRNAs in human disease

Non-communicable diseases, such as cardiovascular disease, diabetes, or cancer, have a major impact on the mortality rates worldwide. In parallel, efforts are made to implement the concept of healthy aging or regenerative medicine [76]. CircRNAs, due to their wide spread function, have impact on all the reminded sectors.

circRNAs in cardiovascular disease

circRNAs are an important effector of the cardiovascular system and are involved in the both physiological and pathological states (Table 1). Circ-Foxo3 is overexpressed in the cytoplasm of aged mice and involved in the regulation of cardiac senescence and myocardial protection [1, 70]; meanwhile, cANRIL is correlated with atherosclerosis risk [77]. CircANRIL binds a 60S assembly factor of ribosomal RNA and it stops rRNA processing, which activates the p53 pathway. In this way, atheroma cells enter apoptosis and are also subjected to a slower proliferation rate [58]. Another important transcript is represented by ciRNA HRCR (heart-related circRNA) that has a protective role in the case of pathological hypertrophy and heart failure [78].

In mice, mmu_circRNA_006636 is significantly downregulated after infarct injury. This circRNA binds to the Dnmt3B and impairs the methylation of Pink1 promoter, leading to its overexpression. Pink1 leads to FAM65B phosphorylation and blocking of autophagy in cardiomyocytes [79].

Table 1 Relevant examples of circRNAs' implication in cardiovascular disease, diabetes, aging, and regenerative medicine

Pathology	cirRNA	Biological function	Biological role	References
Myogenesis	Circ-ZNF609	Translated in protein	Regulation of myoblast proliferation	[64]
Myocardial infarction	Cdr1as (or CiRS-7)	Sponging of miR-7	Expression modulation of PARP and SP1	[1]
Cardiac senescence/cardiomyopathy	circ-FOXO3	Sponging of miR-7	Senescence and stress response factors (ID-1, E2F1, FAK, and HIF1α)	[70]
Heart failure and pathological hypertrophy	HRCR	Sponging of miR-223	Enforced expression of HRCR in cardiomyocytes and in mice displays attenuated hypertrophic responses	[78]
Atherosclerosis	ANRIL (or cANRIL)	–	Polycomb group (PcG)-mediated repression of INK4a/ARF locus	[77]
Cardiac failure	mm9-circ-012559	miR-223	By sponging miR-223, it impairs cardiac hypertrophy	[78]
Diabetes	Cdr1as (or CiRS-7)	Sponging of miR-7	Insulin signaling pathway; targets: Myrip and Pax6	[81]
Diabetes	circHIPK3	Sponging of miR-124 and miR-338	Regulators of β-cell functions in normal and disease conditions by targeting important pancreatic β-cell genes (Slc2a2, AKT1 and MTPN)	[80, 82]
Diabetic retinopathy	Circ-0005015	Sponging miR-519d-3p	Endothelial cell proliferation, migration, and tube formation by increased MMP-2, XIAP, and STAT3 expression	[83]
Immune senescence	Circ-RNA100783	miRNA sponging, alternative splicing, splice variation, and association with phosphoprotein	Target CD28-related CD8(+) T, affecting cell aging	[84]
Ovarian aging	circDDX-10	Sponging of miR-1301 and miR-466	circDDX10-miR-1301-3p/miR-4660-SIRT3 axis related with the ovarian senescence	[85]
Regenerative medicine (diabetes and atherosclerosis)	circ-Amotl1	Sponging for miR-17	*Accelerates* wound healing, by inhibiting miR-17-5p and increasing expression level for fibronectin, Dnmt3a, and Stat3	[86]

circRNAs in diabetes

With fast worldwide aging, the incidence of diabetes and its complications, such as diabetic retinopathy [80], are growing. In this context, circRNA molecular mechanisms offer valuable information (Table 1). In an animal models for type I and type II diabetes, it was demonstrated that circRNAs are important regulators β-cell dysfunction. ciRS-7/CDR1 acts as a miR-7 sponge [81]. CircHIPK3 functions as a ceRNA for miR-124 and miR-338, and it targets the expression of relevant pancreatic β-cell genes, such as Slc2a2, AKT1, and MTPN [82]. Diabetic retinopathy is an important issue in the most of the diabetes patient; there are different circRNAs with altered expression in this condition. Within all, circ_0005015 is a potential biomarker of this disease [83].

CircRNAs in aging and regenerative medicine

CircRNAs accumulation is considered as a hallmark of aging [87]. Although the process of aging is not considered a pathological state, it is connected with a wide range of diseases. The regenerative medicine sits at the opposite side by being more active in a young organism. CircRNAs have an ontogenic shift in their expression profile [88], which is why they are of extreme importance for aging and regenerative studies [89].

The accumulation of circRNAs in the neurons of aging animals is a common event. This is correlated with a decrease in the translational level of genes necessary for physiological function of the cell. The underlying reason for this probably consists in a reduced level of normal splicing and overgeneration of circRNAs [90].

In *Caenorhabditis elegans,* circRNAs accumulate all over the organisms. The deregulated circRNAs are composed of genes related to the life span of the animal. In addition, from 1166 circRNAs identified, 575 consist in de novo sequences. The accumulation of the circular form of transcripts was not done at the expense of linear forms [91]. Other study observed the accumulation of circRNAs in aging neurons accountable for decreased cellular proliferation rate, lower exosome generation potential, and a higher capacity of RBP stabilization of circRNAs [87].

The aging of ovaries show a change in the circRNA profiles, with a total of 194 upregulated circRNAs and 207 downregulated circRNAs. CircDDX-10 sponges either miR-1301 or miR-466 and it leads to the overexpression of SIRT3, a protein involved in epigenetic gene silencing [85].

CircRNAs are important regulators of cell senescence [70, 84]. For instance, circRNA100783 has a key role in chronic CD28-associated CD8(+)T cell aging [84]. Circ-Foxo3 promotes cardiac senescence via cytoplasmic entrapment of transcription factors related to stress and senescence responses [70].

In the brain of aging mice, circRNAs cargo increases, while the linear form has an approximately constant expression. Some of the upregulated circRNAs are: circAnkib1, circZfp609, Circ-Rims2, Circ-dac4, Circ-NFATc3, Circ-Top1, and Circ-Mtf2 [92]. In *Caenorhabditis elegans,* it was again found a global accumulation of circRNAs in older organisms. A total of 797 circRNAs are upregulated, with some of them presenting over a 40-fold increase in the expression level [91].

CircRNAs have a role in the regenerative medicine, especially for stem cell differentiation. The induced pluripotent stem cells (iPSC) have a lower expression of circALPK2, circCACNA1D, circSLC8A1, and circSPHKAP, when compared to differentiated cardiomyocytes [93]. Other study demonstrated that ciRNAs have the capacity to delay or impaired wound healing, particular for the case of patients with diabetes and atherosclerosis. This is the case of circ-Amotl1 that interacts with STAT3, which promotes the nuclear translocation and overexpression of Dnmt3a, with further impact on wound healing through the stimulated proliferation, migration, adhesion, and survival of epithelial cells at the wound site [86].

CircRNAs in cancer

CircRNAs are constantly found at deregulated levels in malignant pathologies with the potential of further use for diagnosis/prognosis tools or therapeutic target.

CircRNA-406483 and circRNA-404833 are overexpressed and the circRNA-001640, circRNA-006411, and circRNA-401977 are underexpressed in human lung cancer tissue versus normal one [94]. The CDR1-derived circRNA is a miR-7 sponge, with implication in multiple pathologies [1, 81, 95–97]. Inhibition of CDR1 is associated with impairment of colorectal cancer progression by increased expression level of miR-7 and positive regulation of epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor 1 (IGF-1R) [98]. Overexpression of CDR1 is correlated with hepatic microvascular invasion in hepatic cancer [99]. Functional studies demonstrate that CDR1 has oncogenic role [100], by stimulating cell proliferation through the modulation of EGFR [101], CCNE1 and PIK3CD [100].

Endothelial circRNAs: cZNF292, cAFF1, and cDENND4C are involved in the regulation of hypoxia [102]. Another circRNA, circDENND4C, is upregulated in breast cancer. This circRNA interacts with HIF1α and promotes cancer cell proliferation [103].

The ANRIL locus in the genome is transcribed both as a linear long non-coding RNA and as a circRNA. It is observed that ANRIL, in linear form or circular form, regulates INK4/ARF gene expression. The change in form is correlated with disease risk via chromatin interaction at 9p21

locus [104], gastric cancer via interaction with TET2 gene [105], non-small cell lung cancer (NSCLC) by silencing KLF2 and p21 expression [106], and hepatocellular carcinoma via epigenetic silencing of KLF2 [107].

CircMTO1 suppresses hepatocellular carcinoma (HCC) progression acting as a sponge of oncogenic miR-9. It has a prognostic role in this disease, where a low expression level predicts a reduced survival rate [108]. In bladder cancer, circMYLK is upregulated, being involved in the regulation of VEGFA expression by acting as a ceRNA for miR-29a [109]. Further details regarding the role of circRNAs in various tumoral pathologies are found in Table 2.

Detection of circRNAs

The detection of circRNAs has reshaped the view of basic and translational research. The circRNAs do not have a poly-A tail and are not capped, which is why they cannot be quantified with the oligo dT enrichment method. Instead of this, scientists can use enzymatic digestion approaches using exonuclease (RNAase R), enzymes that destroy most of the linear transcripts.

The rapid progression of high-throughput RNA sequencing (RNAseq) approaches, sustained by novel bioinformatics tools and databases, allows the quantification of circRNAs in an accurate manner, leading to new insights into to the expression level and biological significance of these transcripts. These procedures for circRNAs analysis consist in the enrichment procedure using RNA exonucleases, enzymes that are capable to digest linear RNA species but not circular ones. These properties are exploited in the procedures of sample preparation or detection.

Most of the circRNAs' next-generation sequencing analysis is based on *in house* pipelines and bioinformatics analysis. These methods allow the identification and characterization of thousands of different circRNAs [118].

Common RNAseq protocols introduce technical artefacts that can result in spurious identification of circRNAs isoforms. It is proven that technical artefacts can be introduced during the ligation and reverse transcription steps of RNAseq library preparation. In general, during most of the sequencing protocols, the libraries are prepared with or without exonucleases treatments (RNase R) followed by ribosomal RNA (rRNA) depletion and complex bioinformatics analysis for the evaluation of the expression level and interaction with coding or non-coding RNAs and proteins. CircRNAs are identified from the RNAseq data as reads having apparent splice junctions that couples the end of a split read fragment to the start of a downstream or an upstream fragment [119]. In both cases, the sequencing of the amplification products is mandatory to certify the accuracy of detection, and to prevent the false-positive detection or improper quantification [118, 120].

Arraystar is the most common circRNAs microarray platform, based on an adapted protocol that uses RNase R treatment and a specific circular junction probe design to achieve a high sensitivity and specificity through enriched circRNAs' fraction [121]. Microarray can be considered as a robust tool for circRNAs profiling, being able to evaluate over 80,000 circRNAs in cervical tumors versus normal tissue and over 18,000 circRNAs in cell-free plasma samples using the Capital Bio Technology Human CircRNA Microarray (version v2.0) with two types of probes with different length (30 nt and 20 nt) [122]. Microarray is proved more efficient and accurate than RNAseq for circRNA profiling; for example, in the case of cervical cancer, around 80,000 circRNAs are identified and 25,000 of them are differently expressed in cervical tumors versus matched normal tissues [122].

Each of the novel cirRNAs identified by RNAseq needs to be validated through qRT-PCR and Northern blot [123]. qRT-PCR is a simple and informative technique. The cDNA synthesis is based on 'outward-facing' and 'opposite-directed' primers [124]. When they anneal to the circRNAs, their 3′ ends face away from each other. RT-PCR can give false-positive detection and improper quantification [118, 120]. To certify the accuracy of the scrambled junction and to avoid template switching or trans-splicing, the sequencing of the amplification products is mandatory [120] The qRT-PCR protocol using divergent primers has the advantage of targeting circRNA back-spliced junction sequence. In this case, only the circRNAs is amplified and not the equivalent linear RNA sequence [123].

Northern blot technique uses the migration of the linear RNAs, along with different circRNAs resulting from *trans*-splicing or tandem duplication. A specific hybridization protocol that can identify the back-spliced junction is required [47]. It is a very usefully approach used for the case of validation of primary structure predicted by bioinformatics tools, with a particular interest when is focused on the differentiation from the other transcripts [47].

Fluorescent in situ hybridization allows the evaluation of cell- and tissue-specific circRNAs expression based on the specific design of fluorescent probes complementary to the back-spliced junctions; these are further connected to high-resolution microscope [125]. Another technology is using the principle of targeting miRNAs using a fluorescent probe compatible for microscopic evaluation; this could be also applied to counteract linear transcripts expressed from the same gene locus as circRNAs [47, 125]. Table 3 presents the main techniques used for circRNAs detection and quantification together with their advantages and disadvantages.

Table 2 Relevant examples of circRNAs implication in cancer

Pathology	Name	Biological function	Biological role	Refs.
Colorectal cancer	Cdr1as (or CiRS-7)	Sponging of miR-7	Upregulated in tumor tissue, promotion of tumorigenesis and invasion Targets: EGFR and IGF-1R	[98]
	CircITCH	Sponging of miR-7, and miR-20a	Wnt pathway; inhibition of c-Myc and cyclinD1 expression	[110]
	Circ-001988	–	Potential biomarker for diagnosis	[17]
	circRNA-001569	Sponging of miR-145	Proliferation and invasion via regulation of E2F5, BAG4 and FMNL2	[18]
Hepatic cancer	Circ-0001649 and circ-0001649	–	Tumorigenesis and metastasis of HCC; potential biomarker in HCC	[111]
	cANRIL	–	Regulation of cell apoptosis via epigenetic silencing of KLF2	[107]
	Cdr1as (or CiRS-7)	Sponging of miR-7	Risk factor for hepatic microvascular invasion; Targets: EGFR, CCNE1, and PIK3CD	[99–101]
	circMTO1	Sponging of miR-9	Downregulated, inhibition of p21, promotion of cell proliferation, and invasion	[108]
Gastric cancer	cANRIL	–	Regulation of INK4a, INK4b and ARF, promotion of cell proliferation and colony formation on in vitro studies via TET2	[105]
Esophageal squamous cell carcinoma	CircITCH	Sponging of miR-7, miR-17, and miR-214	Wnt/β-catenin pathway	[112]
Lung cancer	CircITCH	Sponging of miR-7 and miR-214	Wnt/β-catenin pathway, tumor suppressor role	[113]
	ANRIL (or cANRIL)		Cell proliferation and apoptosis inhibition by silencing of KLF2 and p21 expression	[106]
	Circ-0000064	–	Cell proliferation and metastasis; CircRNA knockdown leads to decreased levels of Caspase-3/9, Bax, p21, CDK6, Cyclin D1, MMP-2 and MMP-9, and increased levels of Bcl-2	[114]
Breast cancer	circ-FOXO3	Predicted as sponging element for miR-22, miR-136*, miR-138, miR-149*, miR-433, miR-762, miR-3614-5p and miR-3622b-5p	Cell proliferation and autophagy, tumorigenesis, and invasion; Targeting of p53 and MDM2; Inhibition of cell cycle progression by binding to p21 and CDK2	[45, 69, 115]
	circ-FOXO3	Protein decoy for CDK2, p21, ID1, E2F1, FAK, and HIF-1α	Cell cycle progression and cell senescence	[69]
Bladder cancer	circRNA-MYLK	Sponging of miR-29	Increased circRNA-MYLK activates cell proliferation, angiogenesis, and metastasis	[109]
	CircITCH	Sponging of miR-17/miR-224	Downregulated, correlated with unfavorable prognostic; regulation of cancer progression via p21, PTEN expression	[116]
Ovarian cancer	ciANKRD52	Transcription regulation	Positive regulator of Pol II transcription	[49]
Glioblastoma		–	Downregulated, regulate cell proliferation	[117]

Table 3 Summarisation of the principle, advantage, and disadvantages of the main detection methods used for quantification of circRNAs

Method	Principle	Advantages	Disadvantage	References
Next-generation sequencing	Exonuclease-based enrichment approaches followed by sequencing	Efficient tool for discovery novel cirRNAS and Profiling The disponibility of non-polyadenylated transcriptomes permits genome-wide identification of thousands of novel circRNAs	Complex bioinformatics algorithm for detection and mapping Bioinformatics analysis is under research	[118]
Microarray	Implies purification of circular RNA (digestion of linear RNA) followed by hybridisation using random RNA primers	Efficient profiling tool; high sensitivity	Requires RNase R treatment; does not allow de novo discovery of circRNAs	[122]
qRT-PCR	RNA digestion, linearization cDNA synthesis and amplification using outforward primers or divergent primers	Used for validation; low price	Template switching products	[123]
Northern blot	Validation method, it implies the use of glyoxal agarose gel, detection using digoxigenin and junction probes	Used for validation; High specificity Visualize circRNAs without artefacts	Require treatment with RNase R and RNase H and purification	[126]
High-throughput sequencing of immunoprecipitated RNAs	Paired-end chemistry for cross-junction fragments	Used for interaction with protein	High cost, requiring deep sequencing	[127, 128]

Databases for circRNAs

Following the development of new high-throughput sequencing technologies, numerous databases were produced, allowing advancement of genomics, transcriptomics, proteomics, and metabolomics. In this way, a promising perspective was created for a more precise evaluation of the expression level of ncRNAs, including circRNAs [129]. The novel circRNAs need to be integrated in a database that allows the summarization of expression pattern for these sequences, integrating in the same time their main isomorphic forms with the exact sequence and the genomic annotation. One of the pioneers in this field is StarBase v2.0 [130, 131]. In Table 4, there is a summary of the key databases/online tools used for circRNA analysis, as well as their advantaged/disadvantages.

CircBase is a complex database that comprises the genomic localization and also the transcribed RNAs along with the information related to the in silico predicted spliced form, the overlapping coding transcripts; it is also connected with the literature data related to the expression level of the circRNAs [132]. Similar to CircBase, Circ2Traits attempts to bring together information about circRNAs that include data from experimental analysis of these RNAs, creating a comprehensive platform of circRNAs associated with human pathologies [133, 134]. CircNet is developed for continuously update with novel circRNAs, providing the genomic annotation together with different isoforms and interaction patterns with coding and non-coding transcripts [129].

There are several softwares used for circularization prediction of transcripts based on RNASeq data. The most important concern when it comes to choosing such a software package is the overestimation of circRNAs generated inside a cell. The CIRI software can generate up to 68% false-positive results, while the CIRCexplorer is the best predictor of circRNAs [135, 136]. This result may be reasoned by the fact that these software packages require the use of gene annotation [135, 137]. Other prediction tools consist in CircMarker [138], PcircRNA_finder [139], find_circ, circRNA_finder, MapSplice, and CirComPara, which focus on the evaluation of abundance and significance of circRNAs in particular cell types [135, 140]. The cancer-specific circRNA database (CSCD) is focused on malignant pathologies [141], while Tissue-Specific CircRNA Database (TSCD) is designed to bring light on circRNAs roles in organ development and tissue-specific disorders [142]. PcircRNA_finder is a software developed for circRNAs' prediction in plants [139]. The most commonly encountered challenge is the overprediction of circRNAs from the RNASeq data.

CircInteractome is a web tool able to evaluate the interactions of circRNAs with RBPs or different coding and

Table 4 Examples of databases and software for circRNAs evaluation

Name	Database/software	Role	Pro	Cons	References
StarBase v2.0	Database	MiRNA–ceRNA, miRNA–ncRNA and protein–RNA interaction networks, provide large-scale CLIP-Seq data	Accurate multi-level prediction of circRNA interaction; row data available	Low detection of isoforms	[130, 131]
Circbase	Database	Data based on circRNA structure and genomic localization, public data sets	Easy to use Contains data from a variety of species Allows detection of overlapping results	Does not contain data about viroids The expression pattern of ciRNAs is not given; the miRNA interactions are not included; low detection of isoforms	[131, 132]
Circ2Traits	Database	Wide knowledgebase of human circRNAs related with disorders or some specific characteristics, under development	Allows genome-wide circRNA interaction with disease associated mutations and miRNAs	It does not include data from other species	[131, 133, 134]
CircNet	Database/Web tool	Provides tissue-specific circRNA expression pattern and presents the relationship between circRNAand miRNA gene	It includes regulatory network Expression data for a high number of loci	No information regarding circRNA interaction with RBP or other types of circRNAs	[129, 131]
CIRI	Online tool	Efficient and unbiased algorithm for circRNAs	Highly sensitive	Generates high number of false-positive results	[136, 145]
CIRCexplorer2	Database/Online tool	Integrative NGS database for circRNAs; comprehensive and integrative circRNA analysis toolset	Accurate prediction De novo identification of circRNAs Balanced sensitivity/precision	Unable to detect circRNAs in case of modified exon boundaries	[21, 137, 145]
CircMarker	Online tool	Accurate algorithm for cirRNAs' detection	Accurate prediction	Does not allow de novo identification Some circRNA with intronic junction cannot be detected	[138, 146]
CSCD	Database	Database for cancer-specific circRNAs	It predicts miRNA-binding sites and the ability of circRNA translation	Limited number of included circRNAs	[141]
TSCD	Database	Tissue-specific data base for human and mouse	Included data was experimentally validated	Data limited to human and mouse Limited number of included circRNAs	[142]
PcircRNA_finder	Database/online tool	Prediction tools for plant circRNA	Higher sensitivity and precision for plant specific circRNAs	Limited to plants Does not include ciRNA interactions	[139]
CircInteractome	Database/web tool	Exploring the interaction with miRNA and RBPs	Provides design of divergent primers specific to circRNAs and also circRNA-based siRNA design It integrates data from circBase, StareBase 2.0	Limited ability to predict interactions	[44, 131, 143]

non-coding RNA transcripts. It also includes information regarding the presence of internal ribosomal entry sites (IRESs), primers for circular transcripts, and siRNA design tools for circRNAs targeting [44, 143].

In line with the latest studies regarding the protein-coding capacity of circRNAs, a new database, circRNADb, was developed. This database includes: an estimation of the circRNAs capacity to encode for proteins, the confirmed results from mass spectrometry analysis, and a tissue-specific pattern of circRNAs [65]. The exogenous circRNAs are presented as robust and stable protein expression in eukaryotic cells, as alternative to linear mRNA [144]. These are only some pioneers' studies that need to be sustained by additional information.

The increase in sequencing depth allows the identification of novel circRNAs by RNAseq. This information, once integrated in public databases, can be used for the generation of specific probes for microarray or other specific determination methods [122]. However, the main problem of these databases is the restrain knowledge related the role of the altered circRNAs and the interconnection of circRNA-miRNAs/lncRNAs-mRNA [147] integrated in interconnected networks of several database.

Circulating circRNAs as disease biomarkers

Exosomes are exocytic microvesicles (30–100 nm) that are released by both healthy and pathological cells to facilitate the communication between them. These vehicles have a cell-type specific cargo, comprised of proteins, DNA, RNAs—mRNA, miRNAs, lncRNAs, lipids, and others [148]. Recently, it was demonstrated that exosomes also contain circRNAs [149]. Li et al. identified over 1000 circRNAs species in human serum exosomes (exo-circRNAs). Their length is mostly under 1000 nucleotides [148]. As follows, the circRNAs act as long-distance regulators, circulating in the body fluids more often in the exosomes than as a free form [150, 151].

CircRNAs are generally expressed at very low levels, but accumulate in the tissue to detectable level due to their high stability [152]. This allows them to be kept at room temperature for 24 h, with no observed modification in the expression level [149].

The exact mechanism regulating the loading of circRNAs into exosomes remains elusive [150]. The circRNAs are enriched in exosomes compared to the cell of origin. In addition, the sorting of circRNAs into exosomes may be regulated by changes in the associated miRNA levels [149].

Due to the high proliferation capacity and increased metabolism of tumor cells, the exosomal circRNAs are isolated in high concentrations. Still, the level of some circRNAs is more reduced in tumor tissue than in the normal one [42, 153]. For instance, circ_002059 is downregulated in gastric cancer tissues, when compared to normal tissue [154].

The ability of circRNAs to act as sponges for multiple targets, including miRNAs, proteins, and possibly lncRNAs, could be used in experimental strategies directed toward cancer inhibition. The synthesis of circular artificial sequences able to impair different oncogenic miRNAs in a simultaneous act could become a highly efficient therapeutic strategy used for the impairment of compensatory mechanisms in cancer.

If an artificial circRNA could sponge, at the same time, various oncogenic miRNAs associated with independent signaling profiles, the chance of resistance is significantly reduced. A superior level of cancer therapeutic modulation could be represented by hybrid circRNAs able to simultaneously bind oncogenic miRNAs and oncoproteins of the same pathway [155]. This strategy is especially important in the context of drug resistance in cancer, one of the main issues responsible for increased mortality rates [156–159].

Engagement of these approaches in the preclinical research could result in a more extended modulation of non-coding and proteomic profiles, decreasing significantly the change of cancer cells to activate secondary survival pathways. The design of artificial circRNAs should target several upstream and downstream elements from a signaling pathway, thus preventing the development of secondary resistance (Fig. 3).

The therapeutic potential of circRNAs lays mainly in their ability to sponge microRNAs (Fig. 4). Some circRNAs could be subjected to inhibition, because they are overexpressed in cancerous cells and sponge the tumor suppressors' miRNAs; as in the case of circGFRA1 and miR-34a [96] or circUBAP2 and miR-143 [160]. There are a number of in vitro studies based on this therapeutically strategy. Circ-SMARCA5 is an androgen-induced overexpressed circRNA in prostate cancer and its inhibition by siRNA targeting the back-splicing site leads to cell apoptosis and inhibited cell proliferation [161]. CircRNA-MYLK functions as a ceRNA by binding miR-29a and allowing the expression of VEGFA. The silencing of this circRNA was also proposed for the treatment of bladder cancer [109]. cZNF292 is a hypoxia-induced circRNA, whose expression silenced by siRNA leads to suppressed proliferation and angiogenesis in glioma [162].

Other circRNAs sponge oncomiRs and their overexpression is also proposed as a therapeutic option. In hepatocellular carcinoma, circMTO1 sponges miR-9 and allows the expression of p21. siRNA targeted toward this circRNA promotes tumor growth, invasion, and proliferation [163].

Circular RNA-ITCH (circITCH) has also therapeutic potential by downregulating the Wnt/β-Catenin pathway [113] in: colorectal cancer [110], bladder cancer [116], or esophageal squamous cell carcinoma [112]. Circ-FOXO3

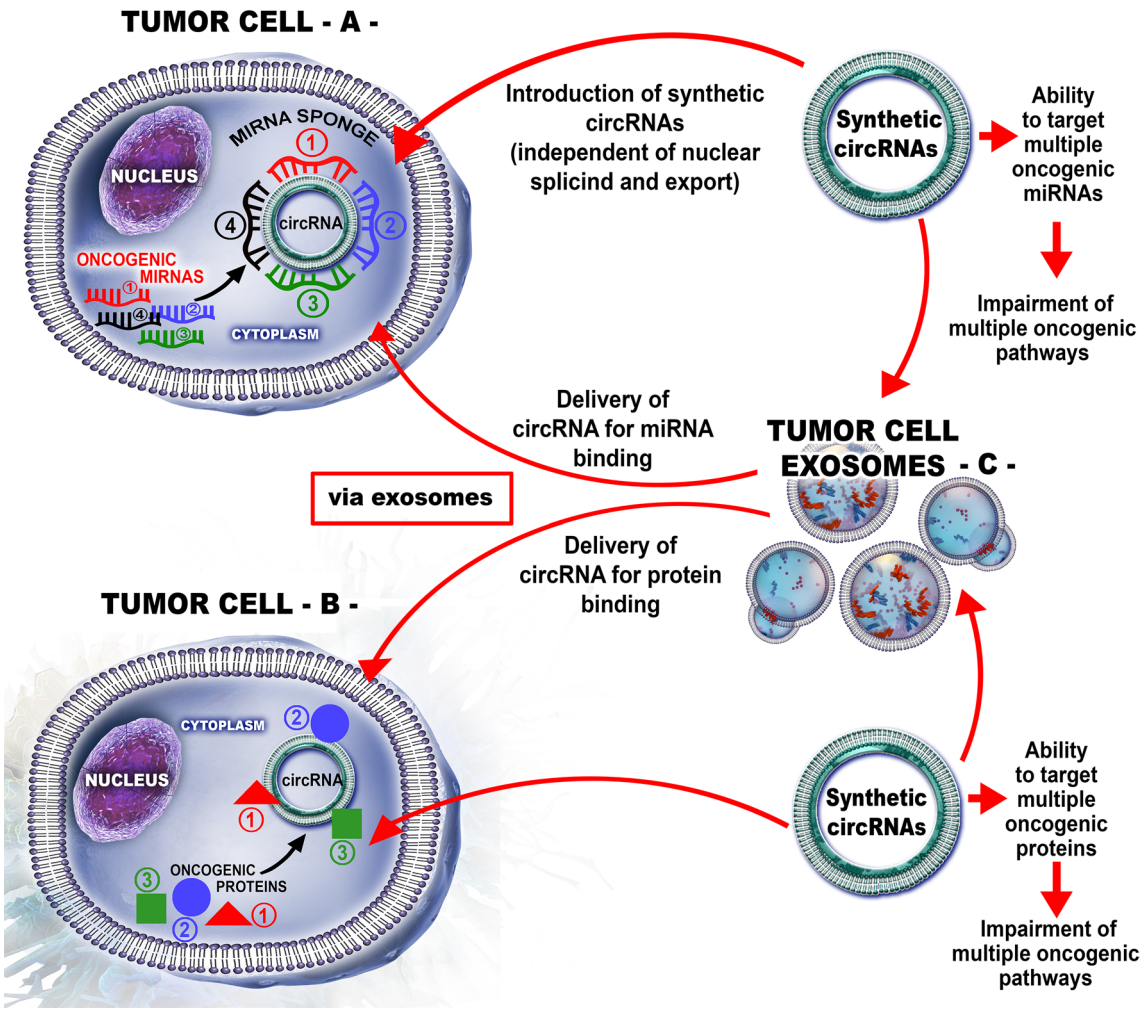

Fig. 3 Therapeutic value of synthetic circRNAs. **a** The synthetic circRNAs can be design in such a way that it will be able to target multiple miRNAs with consequences toward impairment of multiple oncogenic pathways. Synthetic circRNA can be delivered as a single agent. Inside the cell, this circRNA will sponge multiple microRNAs; **b** a second option consists in synthetic circRNAs able to target multiple proteins leading to the impairment of multiple oncogenic pathways. Once delivered inside the cell, the oncogenic proteins will be sponged and their activity suppressed; **c** both types of circRNAs can be loaded in tumor cell exosomes which will enhance their targeting efficiency and amount that can be delivered

entraps the CDK2 and p21 proteins, thus, impairing cell cycle progression in cancer [69]. Circ-FOXO3, when transfected into breast cancer cells, causes a decrease in tumor size and it enhances cell death [75, 164].

On top of using circRNAs as therapeutic options, they can also be used as vectors, for microRNAs or proteins [155]. One important challenge that may come in the future development of circRNA-based therapies is the lately discovered fact where exogenous circRNAs are recognized by RIG-I as non-self-molecules through an unknown mechanism. The exogenous circRNAs, in contrast to the endogenous ones, do not possess the RNA associated proteins: U1, U2, and U4/U6/U5 tri-snRNP subunits, EIF4A3, MGN2, RNPS1, DDX39B, THOC4, and XPO5. Moreover, the intracellular delivery of exogenous circRNA gives rise to a rapid increase in the expression level of various inflammation-associated genes [155, 165]. Even if cancer-derived exosomes have an increased tropism for malignant cells, there is also the possibility of targeting healthy ones, resulting in phenotypic transformation or induced cell death. In these sense, hybrid strategies need to be taken in consideration, combining different delivery perspectives from the nanopharmacology domain [166].

Conclusions

CircRNAs are key regulatory elements in all the stages of cellular development. Some of them have the status of housekeeping transcripts, while others are tissue-specific.

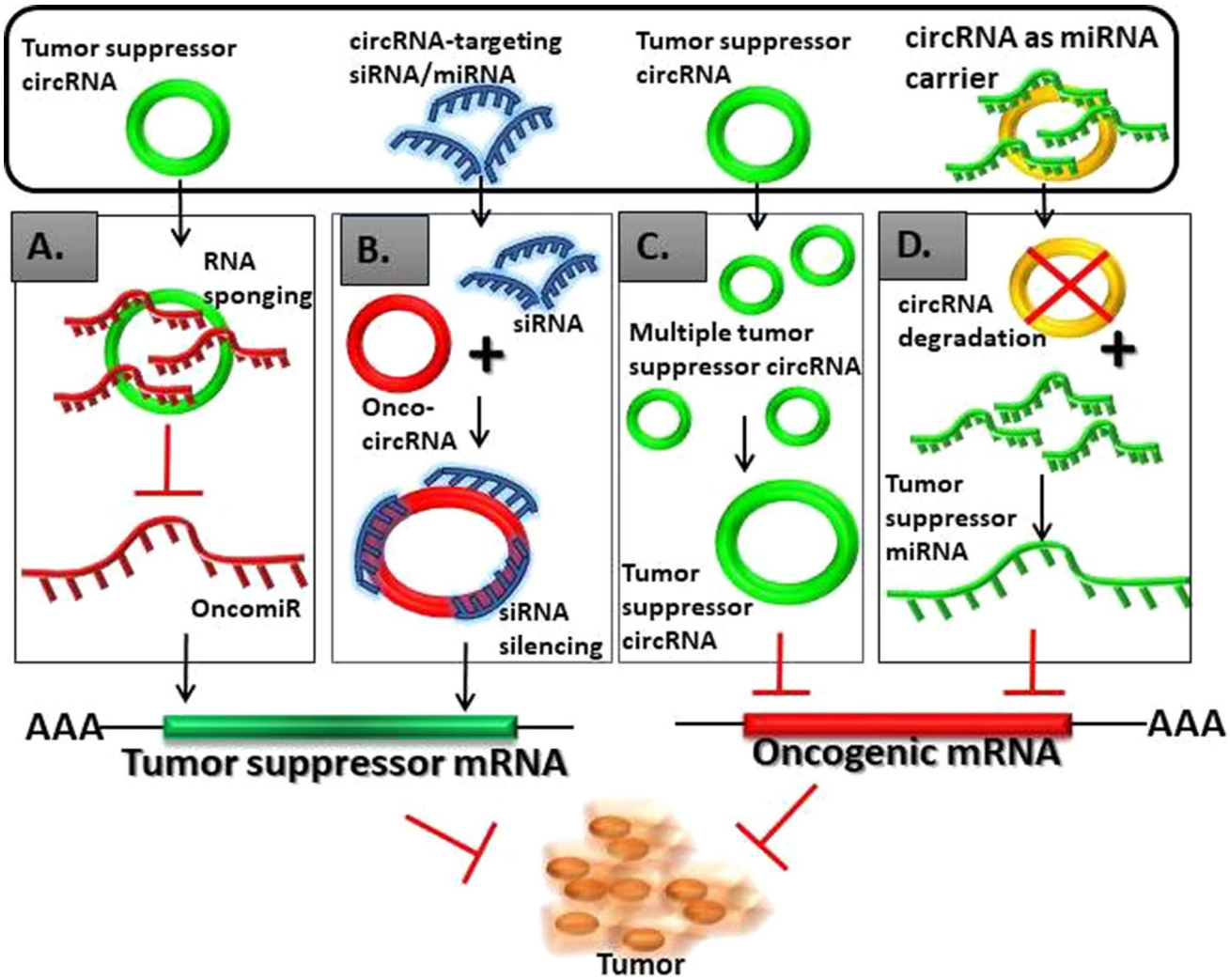

Fig. 4 The major strategies for circularRNA therapy. **a** A tumor suppressor circRNA inside the cell will sponge oncomiRs, resulting in the upregulation of tumor suppressor mRNAs. **b** In the intracellular environment, siRNA can target and repress the oncogenic circRNA and the upregulation of tumor suppressor mRNA. **c** Inside the cell, the number of tumor suppressor circRNAs is restored and these can directly inhibit the oncogenic mRNAs. **d** circRNAs can also function as miRNA delivery systems, where tumor suppressor miRNAs can further repress the oncogenic mRNA. All of the above-mentioned circRNA therapeutic strategies result in the mRNA-mediated tumor suppression

Moreover, during pathological states, the expression of circRNAs is shifting toward a differential one, the fact that is transforming these sequences in important diagnosis and prognosis tools, but also therapeutic targets. Moreover, the main known role of circRNAs, namely miRNAs sponging, highlights the idea of using these circular transcripts as inhibitory vectors for oncogenic overexpressed miRNAs. Moreover, similar techniques could be used for therapeutic strategies to eliminate the harmful activity of aberrantly overexpressed circRNAs. CRISPR/Cas9 has emerged as an important technique for the investigation of gene function and also therapeutic strategies [167, 168]. For the case of, circRNAs complete depletion via CRISPR/Cas9 of the genomic corresponding region could offer specific insights within the role of circRNAs in cellular homeostasis and also development toward pathological states.

The interference with important regulatory processes of mRNA metabolism on many levels: transcription, splicing, mRNA turnover, and translation, makes circRNAs key molecules within evaluation of pathological processes, with further implication in the possible novel clinical management tools. The utility of these circRNAs as circulating molecules in diagnosis procedures can be justified by the first clinical trials (NCT03334708) launched [169]. This study evaluates a panel of circRNAs, along with other molecular markers (proteins and proteases, functional DNA repair assays, exosomes, stromal elements, and circulating tumor DNA) for the diagnosis of the early stage pancreatic cancer.

The main problem remains the optimisation of the detection and quantification methods, where the research is still in the early phases. Even so, circRNAs hold great promises due to their complex regulatory capacity in different stages of development and also differential profiles in pathological states.

Author contributions CB wrote the paper; A-AZ participated for the introduction part and for figures concept; DG assisted for the table preparation and wrote the final part related to the circulating cirRNAs as biomarkers. AO was responsible for the figure design and for the part related to data-based and programs for circRNAs application. IBN was the design of the study, final correction of the manuscript. All the authors assisted in the preparation of the manuscript and editing and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest Authors have no financial and non-financial competing interests to be declared.

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