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Review

Dysregulation of circular RNAs in inflammation and cancers

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ABSTRACT

Emerging lines of evidence have shown that the production of the covalently closed single-stranded circular RNAs is not splicing errors, but rather a regulated process with distinct biogenesis and turnover. Circular RNAs are expressed in a cell type- and tissue-specific manner and often localize to specific subcellular regions or organelles for functions. The dysregulation of circular RNAs from birth to death is linked to the pathogenesis and progression of diverse diseases. This review outlines how aberrant circular RNA biogenesis, subcellular location, and degradation are linked to disease progression, focusing on metaflammation and cancers. We also discuss potential therapeutic strategies and obstacles in targeting such disease-related circular RNAs.

1. Introduction

Recent studies have revealed that non-coding RNAs or uncanonical coding RNAs play important roles in the pathogenesis and progression of diseases [1–3]. Among them, circular RNA, which was a mystery decades ago, has become well-investigated in recent years [4–7]. The most remarkable feature of circular RNA is the covalently closed loop structures that lack 5′ and 3′ ends. One type of circular RNA (circRNAs) is mainly produced by the back-splicing of exons in precursor messenger RNAs (pre-mRNAs) (Fig. 1) [8]. Recent studies have shown that the back-splicing of exons is regulated by intronic complementary sequences flanking the exons and facilitated by RNA-binding proteins, such as Nuclear factor 90 (NF90) and NF110 binding to these elements [9,10]. Alternatively, a subset of intron-lariats can escape from debranching and retain the covalently closed structure to form circular intronic RNAs (ciRNAs) [11,12]; while others are produced from the mitochondrial genome with yet-defined biogenesis pathways, such as SCAR, mc-COX2, and mecciRNAs [13–15]. Owning their covalently closed structures, circular RNAs are resistant to linear RNA decay machinery, thereby they are more stable in general than linear RNAs. Nevertheless, emerging studies have shown that circular RNAs can regulate different biological processes and their abnormal expression is related to the physiological and pathological processes of natural immunity, metabolic inflammation, neuronal disorders, and tumors [13,16-18]. Importantly, some exon back-splicing-derived circRNAs have been found to play important roles in multiple life activities and show potential applications for the diagnosis and treatment of diseases [6]. Therefore, understanding the nature of circRNA's biogenesis, metabolism and function is of great importance in fully appreciating their functions and potential applications.

Previous studies have found that circRNAs have different expression profiles in different organs and tissues, suggesting that circRNAs are expressed in a cell type-specific or tissue-specific manner [19]. Moreover, several recent studies have identified circular RNAs in nuclei and mitochondria, implying that they can also be expressed in an organelle-specific manner (Fig. 1) [13,20]. circRNAs located in the cytoplasm can play the role of "miRNA decoy" and regulate the expression lev-

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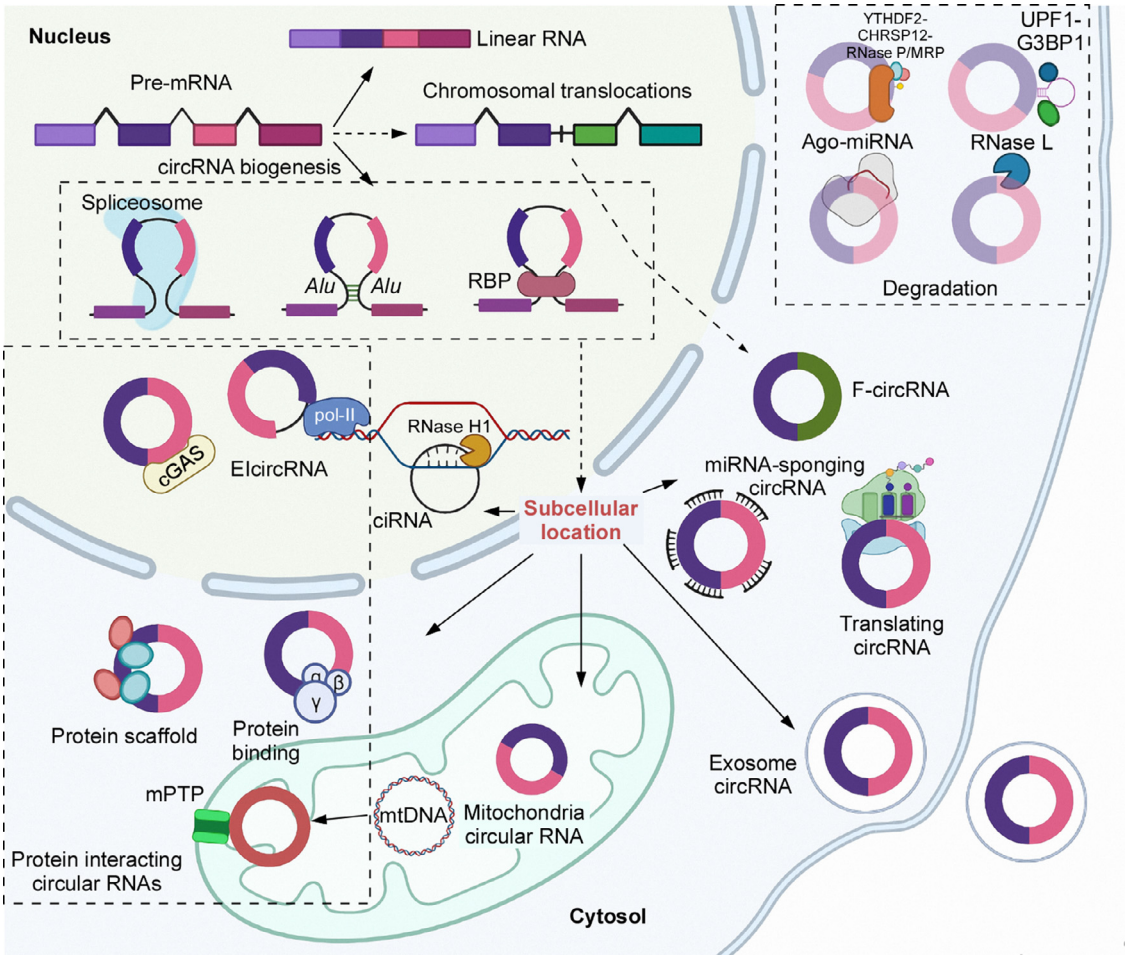

Fig. 1. Circular RNAs from biogenesis to degradation. The biogenesis of circRNA is mediated by spliceosomes, intronic complementary sequences, and RNA-binding proteins (RBP). Chromosomal translocation can lead to the biogenesis of fused circRNA (F-circRNA). After generation, circular RNAs can have different subcellular locations. In the nucleus, EIcircRNA or ciRNA with introns retained participates in gene regulation, while cia-cGAS binds cGAS in an autoimmune response. In the cytosol, circRNA can sponge miRNAs, interact with proteins, or be translated into proteins. Circular RNAs can also locate mitochondria or exosomes, such circular RNAs are highly associated with metabolic homeostasis. At the end of their lifetime, circular RNAs undergo decay according to their sequence modification, secondary structure, miRNA binding, or stress conditions. Made by BioRender (https://biorender.com).

els of miRNA-targeted genes [21,22]. In addition, circRNA also has the function of a "protein decoy" [23]. Recent studies have also revealed that circRNAs can be translated into proteins [24]. In the nucleus, some intron-retained circRNAs are involved in gene expression [20]. As for the mitochondria, we showed that mitochondrial circular RNA, *SCAR*, could regulate mROS output and maintain metabolic homeostasis [13]. These studies showed that the organic and subcellular locations of circular RNAs are essential for their biological functions. The dysregulation of the subcellular location of circular RNAs may contribute to unbalanced cell metabolism.

In this review, we discuss recent findings of diseases related to dysregulation of biogenesis, degradation, and subcellular location of circular RNAs. We also summarize the current understanding of therapeutic strategies related to circular RNA biology.

2. Biogenesis of circRNAs and its dysregulation in diseases

2.1. Computational detection and quantification of circRNAs in various diseases

Considering that most exonic circRNAs are highly similar to their cognate linear transcripts, only reads that span the back-spliced junction site can be effectively identified as circRNAs. Recently, several computational tools have been developed for detecting circRNAs, including find_circ [22], CIRCExplorer [9,25], CIRI [26,27], and MapSplice [28], etc. Most of these methods employ alignment-based strategies to recognize back-spliced junction sites, which may have limited sensitivity and notable false-positive rates. This is because most alignment tools are not designed for the alignment of these non-linear transcripts [29,30]. Most recently, by leveraging long-read sequencing technologies (*e.g.* Nanopore), several workflows and bioinformatic tools have been developed to sequence and reconstruct full-length circRNA isoforms [31–35].

Differential expression analysis is an important way to mine candidate circRNAs that are related to diseases. However, given the generally low expression of circRNAs and the variation of enrichment efficiency and library quality in different datasets, differential expression of circRNAs is a challenging aspect in circRNA studies. Recently, a few algorithms have been developed to directly estimate circRNA expression, some of which can quantify the expression of both circular and cognate linear transcripts [36–39]. Importantly, as single-cell sequencing could potentially help in studies of RNA cellular architecture in human inflammation and cancer in the future, computational tools and single-cell approaches that identify circRNAs at the single-cell RNA level have also been developed recently [40–42]. The comprehensive single-cell RNA profile analysis will help to understand circRNA diversity in diseases, which may identify distinct circRNA-expressing cell subsets and reveal how these subsets affect disease progression. Nevertheless, more

sophisticated statistical models and computational algorithms are still needed to deepen circRNA studies to the isoform-level resolution.

2.2. Physiopathological expression of circRNAs

The back-splicing of circRNAs is carried out by spliceosomes binding to exons and their flanking introns of pre-mRNAs (Fig. 1) [43]. Although the efficiency of back-splicing is much lower than canonical splicing, the produced circRNAs are more stable than linear RNAs and sometimes have a higher circular-to-linear RNA ratio (CLR) [43]. Recent studies have shown that nucleated hematopoietic cells have an averagely high CLR, which may play an important role during hematopoietic differentiation [44,45]. Thus, proper CLR is essential to the cellular functions of circRNAs, and dysregulation of circRNA biogenesis may result in aberrant circRNA expression and thereby be related to disease pathogenesis. For instance, polypyrimidine tract binding protein 1 (PTBP1), a widely studied splicing regulatory factor in the hnRNP family, is predicted to bind 3 sites upstream of the circRNA_001160 formation region on pre-mRNA. Thus, upregulated PTBP1 in glioma endothelial cells causes the excessive generation of circRNA_001160 instead of its linear one, which enhances the blood-tumor barrier and impedes the therapy of glioma [46]. Additionally, HNRNPL can up-regulate circRNA splicing, which is clinically relevant in human prostate cancer [47]. HNRNPM can regulate the generation of mitochondrial circular RNA SCAR, which inhibits the activation of liver fibroblasts in non-alcoholic steatohepatitis (NASH) [13].

The generation of circRNAs also often requires intronic complementary sequences, most of which are Alu repeat elements flanking the circularized exons in the human genome (Fig. 1) [48,49]. Disruption of such intronic pairing causes a complete loss of circRNAs or aberrant circRNA expression. In the study of Xia et al., the knockout of cia-cGAS via Alu deletion using CRISPR/Cas9 in mice led to a decrease in the number of LT-HSCs which was associated with severe anemia and death [45]. Intriguingly, recent studies have revealed that cancer-associated chromosomal translocations gave rise to coincidental intronic complementary sequences flanking the exons of distinct genes and generated fusion circRNAs (f-circRNAs) (Fig. 1), which functioned in acute myeloid leukemia (AML), Ewing sarcoma and lung cancer [50,51].

In addition to the roles of spliceosomes and intronic complementary sequences, RNA binding proteins (RBP) also regulate circRNA biogenesis (Fig. 1) [52]. NF90 and NF110 are found to bind to intronic inverted-repeat Alu elements with their double-stranded RNA binding domains (dsRBDs) and promote back-splicing [10]. Under viral infection, these immune factors are exported to the cytoplasm and induce an antiviral immune response, meanwhile, significantly downregulating circRNAs, suggesting that NF90/NF110 can coordinate with circRNA biogenesis and function in viral infection. In another study, RBP FUS which is associated with the pathogenesis of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia, was found to modulate circRNA biogenesis through binding introns flanking the circRNA-forming exons. Moreover, most circRNAs regulated by FUS were conserved in mouse and human motor neurons, implying links between circRNA production and ALS pathology [17].

Since various factors regulating the generation of circRNA are disease-associated, it is quite important to understand the pathway and mechanism of circRNA biogenesis, especially the similarities and differences between back-splicing and classical splicing reactions and their specific regulators. In addition, how circRNAs maintain a stable CLR in health conditions is also a cutting-edge scientific question that enlightens novel treatments for diseases. Last but not least, it is interesting that some circular RNAs are found to be generated by mitochondrial genes [13,15,53], which lack introns, let alone intronic complementary sequences, so the nature of biogenesis of mitochondrial circular RNAs needs further exploration.

3. circRNA action modes and diseases

After generation, circRNAs seem to act through diverse mechanisms according to their biological functions. In the cytosol, circRNAs can act as microRNA decoys, which can bind microRNAs (miRNAs) possessing complementary sequences to multi-sites in circRNA sequences and then withstand the silencing effect of miRNAs on targeted mRNAs (Fig. 1) [21,22,54]. Some circRNAs are also reported to interact with proteins (Fig. 1). For example, cytosolic circFoxo3 interacts with the cell cycle proteins cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (p21) to form a ternary complex that inhibits CDK2's function in cell cycle progression [55]. This circRNA is also found to bind multi-anti-stress proteins and plays important roles in myocardial cell senescence [56]. In addition, many cytosolic circRNAs are found to have unique ORFs and can be translated into proteins [57–61]. In the nucleus, circRNAs are mostly involved in regulating gene transcription, alternative splicing, and chromatin loops (Fig. 1) [11,20,62,63]. Despite the uncovering of these emerging roles, the potential functions of a large number of circRNAs remain unknown. Due to the tissue-specific expression manner, circRNA functions diversely in different tissues, so the dysfunction of circRNAs is linked to various systematic diseases. Here, we grouped circRNAs with different biological functions into three types: miRNA-sponging circRNAs, protein-interacting circular RNAs, and protein-translating circRNAs, and discussed the immune metabolic diseases and cancers related to the dysregulation of these functions.

3.1. miRNA-sponging circRNAs

We and others have previously shown that post-transcriptional regulation by miRNAs determines cell fate by coordinating transcriptional factors and controlling their targeted mRNAs [64,65]. Highly abundant circRNAs containing miRNA binding sites can sponge miRNAs and have competing endogenous RNA (ceRNA) function, suggesting that circRNAs can regulate disease progression through sponging miRNAs. The most well-known circRNA CDR1as, which contains more than 70 conserved miR-7 binding sites, was initially found to regulate neuronal development and may serve as a potential biomarker for neurological disorders and tumors [21,22]. MiR-7 is a putative tumor suppressor of colorectal cancer [66]. Researchers found that circCDR1as was significantly upregulated in colorectal cancer patients and blocked miR-7, leading to the activation of miR-7′s targeted genes EGFR and RAF1, which were associated with tumor progression [67]. Moreover, emerging evidence has also shown that a number of circRNAs, working as ceRNAs, could bind multi tumor suppressor miRNAs and regulate a subset of genes linked to the proliferation of cancer cells [68,69]. Increasing oncogenic or tumor-suppressing miRNA-sponging circRNAs have been reported recently, implying the important role of ceRNA function in various cancers [70,71]. In addition, several studies have revealed that some miRNA-sponging circRNAs are associated with metabolic disorders such as diabetes, obesity, and insulin resistance [72–74]. These studies suggest that the dysregulation of the circRNA-microRNA-downstream molecule axis may be prevalent in metaflammation and tumor progression.

However, there are some debates on how common this mechanism is for endogenous circRNAs. According to a paper from Bartel's lab, the theory of miRNA sponge is doubted since most circRNAs contain much fewer miRNA binding sites than CDR1as (more than 70 target sites), circZNF91 (24 target sites), or circSYR (16 target sites) [75]. The low copy number and limited miRNA binding sites of most circRNAs are actually confusing in terms of how they affect cancer progression via ceRNA function [76,77]. Maybe it should be considered that the impacts of reported circRNAs with such modes of action are measurable possibly due to their stability or over-expression in diseases.

3.2. Protein-interacting circular RNAs

RNA-binding proteins also play a critical role in circRNA functions. As many RNA-binding proteins also act as transcription regulators, some nucleus-localized circRNAs can recruit transcriptional factors to the promoter region of the targeted genes and regulate their expression (Fig. 1). Some intron-retained circRNAs (EIcircRNAs) such as circEIF3J and circPAIP2 have been identified to recruit U1 snRNP and pol-II to the promoter of parental genes and enhance gene expression [20]. Another article recently reported that circRNA FECR1 can recruit a demethylase TET1 to the promoter of an oncogenic driver, friend leukemia virus integration 1 (FLI1), leading to the overexpression of FLI1 and promoting the metastasis of breast cancer [78]. Through sponging RNA-binding proteins, circular RNA may play a role in inflammatory signaling pathways. We have recently reported a mitochondrial circular RNA SCAR, which binds the regulator of mitochondrial permeability transition pore (mPTP) to inhibit mROS-mediated fibroblast inflammation in nonalcoholic steatohepatitis (NASH) [13]. A latest study also showed that circMTCL1 binds to C1QBP and subsequently activates the wnt/β-catenin pathway in the advanced laryngeal squamous cell carcinoma (LSCC) [79]. Importantly, some circRNAs are involved in the regulation of immune sensors such as double-stranded RNA (dsRNA)-activated protein kinase (PKR) in systemic autoimmune diseases [16] and RIG-I in viral infection [80,81] as well as cGAS in autoimmune response [45]. In addition to influencing the exterior function of proteins, circRNAs have recently been found to affect proteins' structure as well. Another article reported that circVAMP3 acts as a molecular skeleton to promote the phase separation of CAPRIN1 and inhibits the proliferation of hepatocellular carcinoma (HCC) cells by suppressing c-Myc translation [82]. RNA binding proteins' functions are widely spread through diverse mechanisms and an increasing list of circRNAs has been identified to be involved in the circRNA-protein interaction, which is very often linked to different disease processes as discussed above.

3.3. Translating circRNAs

In recent years, the translation of circRNAs and their therapeutic potential have increasingly gained the attention of researchers [24]. Although circRNA is 5′-m^{7}G cap-independent, researchers found that RNA circles with internal ribosome entry site elements (IRES) can be translated *in vitro* or in cells [83,84]. Abe's lab also found that RNA circles can be translated through rolling circle amplification [85]. Afterward, a group of endogenous circRNAs was found to possess ribosome-protected sites, specific ORFs, IRESs, or m^{6}A RNA modification sites, which strongly suggests that circRNAs are translatable [60,61,86]. Importantly, recent studies have shown that novel proteins produced by circRNAs are involved in human diseases. Zhang, N's research team showed that SHPRH-146aa, FBXW7–185aa, and PINT87aa translated by circRNAs act as tumor suppressors in human glioblastoma [18,58,59]. They also found that HER2–103 encoded by circHER2 is highly expressed in triple-negative breast cancer (TNBC), which is associated with a worse overall prognosis of breast cancer [87]. Another study identified circMAP3K4 with coding potential in HCC patients using bioinformatic analysis and that circMAP3K4–455aa could protect apoptosis-inducing factor (AIF) from cleavage so HCC cells are resistant to cisplatin-induced apoptosis [88]. A latest study also found that the downregulation of circBNC2 in epithelial cells which is translated into ctBNC2 protein to regulate the G2/M cell cycle is associated with human fibrotic kidney and liver [89]. Therefore, the finding of translation of circular RNAs has broadened the traditional understanding of non-coding RNAs or uncanonical coding RNAs. It's also worth exploring whether tumor-specific circRNAs could encode antigen peptides which may elicit anti-tumor immune response.

Undoubtedly, there are also some debates on how prevalent circRNA translation could be. Although recent studies suggested that circRNA translation is very prevalent [86,90], several recent papers have argued that most of these translatable circRNAs are due to artifacts [91,92]. Additionally, due to the noise of mass-spectrometry data, many of the circRNA-encoded proteins reported in the cancer papers may actually be translated from novel linear splicing isoforms [93,94]. Therefore, additional experiments are needed to exclude potential artifacts, especially for unknown proteins produced by circRNAs. It should be noted that circular RNAs are often expressed at a low level and cap-independent translation is inefficient, thus, to what degree endogenous circular RNA translation can give rise to measurable effects should be carefully evaluated [95].

Nevertheless, although the copy number of most circRNAs *in vivo* is low [16], the stability of circRNAs may endow some irreplaceable functions in tissues and cells. Individual circRNAs may even have multiple roles to strengthen their ability to control the progression of diseases [96]. Proper classification of circRNAs according to their biological functions and what characteristics determine circRNA's functions warrant future studies. More importantly, it is necessary to face controversies over circRNA biogenesis and functions and then solve those problems, which require a more accurate and high-tech approach to adapting to circRNA research. Uncovering the nature of circRNA's biogenesis and biological function is of great importance to efficient treatments of diseases.

4. circRNA degradation and diseases

Although circRNAs are resistant to RNA enzymes participating in the degradation of linear RNAs, recent studies have revealed that circRNAs undergo decay by various mechanisms (Fig. 1). For example, circRNA CDR1as has been found to be directly cleaved by miR-671-AGO2 [97]. And long noncoding RNA Cyrano could suppress miR-671 through target-directed miR-7 degradation, leading to the accumulation of circRNA CDR1as in brain cells [98]. Another study showed that *Drosophila* GW182 and its human homologs (TNRC6A, TNRC6B, and TNRC6C) mediated circRNA decay in an AGO-slicer or P-body independent manner [99]. In stress conditions, circRNAs can also be degraded by RNase L, which functions more globally than in an AGO-dependent manner [13,16]. Additionally, some m^{6}A-containing circRNAs can also be cleaved by YTHDF2 (m^{6}A reader protein)-HRSP12 (adaptor protein)-RNase P/MRP (endoribonucleases) [100]. Moreover, circRNAs with secondary structure may undergo decay by UPF1 (RNA-binding protein) and its associated protein G3BP1 [101]. Interestingly, cleaving circRNAs is like a part-time job for the factors mentioned above, leading to low efficiency of compensation for dysregulating circRNA expression under disease conditions. Therefore, uncovering the major pathway of circRNA decay is of great importance to disease treatment.

Steady-state amounts of circRNAs play important roles in the immune system [10,16]. In autoimmune diseases such as systemic lupus erythematosus (SLE), virus infection leads to the activation of RNase L, which degrades great amounts of circRNAs, including circRNAs that serve as endogenous inhibitors for PKR. Then PKR is further activated and moves forward to induce disease progression [16]. Meanwhile, during virus infection, NF90/NF110 is exported from the nucleus to mediate the immune response [10], resulting in decreased biogenesis of circRNAs which subsequently enhances the unbalance of the immune system. Intriguingly, mitochondria-localized circular RNA SCAR can also be degraded by RNase L in liver fibroblasts. Under lipid stress, PGC-1α, which regulates the transcription of SCAR, is suppressed by ER stress mediators, leading to a remarkable decrease in SCAR in NASH. It is important to note that the copy number of SCAR in liver fibroblasts is nearly over 1000 due to high copies of the mitochondrion, suggesting that this circular RNA is stable in normal conditions [13]. In liver fibrosis, RNase L-mediated circular RNA decay may contribute to such a remarkable decrease in SCAR, which in principle would then release immune factors to maintain metaflammation. What's more, accumulating lines of evidence have indicated that m^{6}A deregulation drives tumor progression [102]. As m^{6}A modification of circRNA has been suggested

as a marker for "self" [81], tumor-derived circRNAs modified by m^6A may mark themselves as "self" circRNAs and escape tumor immune responses. Therefore, the inefficient degradation of these m^6A circRNAs may contribute to tumor immune escape. In addition, it is also possible that m^6A modification of circRNAs in tumor cells leads to their decay by the YTHDF2-HRSP12-RNase P/MRP complex and subsequently covers up their immunogenicity. Nevertheless, further investigations are required to elucidate the nature of circRNA degradation.

5. circRNA subcellular location and diseases

The multiple expression profiles of circular RNAs in different organ tissues revealed their cell type- and tissue-specific expression manners [19,103,104]. For example, CDR1as acts as a molecular decoy for miR-7 and miR-671 in brain tissues and maintains the function of the nervous system [19]. Testis-located circSRY plays a role in the reproductive system [105]. circRNA HIPK3 acts as an oncogene in colorectal cancer [106], but it is also considered a tumor suppressor in bladder cancer [107]. In addition to cell type-specific or tissue-specific expression, the latest studies have revealed that circRNAs also display organelle-specific expression, showing specific enrichments in nuclei, mitochondrion, and exosomes (Fig. 1) [13,14,20,45,108-110]. It is of great importance to link the mechanism of circRNAs' subcellular location patterns, modes of action, and disease processions.

5.1. Nucleus-localized circRNAs

Although the back-splicing of exons occurs in the nucleus, most exons-deriving circRNAs are exported to the cytoplasm. However, intron-containing circRNAs such as EIcircRNAs and ciRNAs can stay in the nucleus and interact with transcriptional factors or form R-loops to regulate the transcription of parental genes (Fig. 1) [12]. For instance, cis-acting circ-CTNNB1 can bind DEAD-box polypeptide 3 (DDX3) in the nucleus and enhance the transactivation of Yin Yang 1 (YY1), resulting in transcriptional activation of downstream genes associated with Wnt/beta-catenin signaling, which regulates tumorigenesis and aggressiveness [111]. Nuclear localized circDONSON can recruit the NURF complex to the SOX4 promoter and initiate its transcription, causing the tumorigenesis of gastric cancer [112]. On the other hand, circHuR detected in the nucleus can prevent CNBP from binding to the *HuR* promoter and suppress gastric cancer progression [113]. In another example, circAnks1a was found to directly bind to the *Vegfb* promoter and act as a regulator for neuropathic pain [114]. In addition to interacting with transcriptional factors and gene promoters, high levels of cia-cGAS in the nucleus could bind the DNA sensor cGAS to protect long-term hematopoietic stem cells (LT-HSCs) from cGAS-mediated autoimmune responses and maintain their quiescent state [45]. Another set of circRNAs in the nucleus is involved in the regulation of tumorigenesis signaling pathways such as the Wnt/beta-catenin signaling [115], making them potential candidates as biomarkers for cancer diagnosis and novel targets for cancer treatment.

5.2. Mitochondrial circular RNAs

Mitochondrion has its own genome, so alternative splicing and back-splicing can also occur in the mitochondria to generate mitochondrial circular RNAs (mito-circRNAs). The evidence of spliceosomes in mitochondria regulating mitochondrial RNA biogenesis also strongly supports this concept [116]. Liu et al. have found hundreds of circular RNAs encoded by the mitochondrial genome (mecciRNAs), some of which may play a key role in the process of mitochondrial protein import [15,53]. However, the biogenesis of mecciRNAs has remained unclear. MtDNA-transcribed circRNAs are also found to be widespread and translatable in plants [117]. It has also been reported that mitochondria-derived circRNAs are associated with the progression and prognosis of chronic lymphocytic leukemia [14]. Moreover, our latest study clarified the important role of mitochondria-localized circRNAs in immune metabolic inflammation, suggesting that mito-circRNAs play important roles in mitochondrial metabolism [13]. In addition to circular RNAs produced from transcribed mtDNA, nuclear-derived circRNA PUM1 is found to localize in mitochondria and mediate oxidative phosphorylation function in esophageal squamous cell carcinoma cell lines [108]. However, the mechanism of this mitochondria-specific expression in diseases remains unknown. It is of great concern to uncover the mitochondria importing pathways of these circular RNAs, which may play essential roles in cell metabolic regulation in immune diseases or cancers. Overall, the findings of mitochondria-localized circular RNAs derived from the mitochondrial genome or nuclear genome call for new therapeutic strategies that target them inside the mitochondria. It is also important to distinguish the circular RNAs transcribed from the mitochondrial genome and those transcribed from the nuclear-mitochondrial segments (NUMTs) to avoid and exclude potential artifacts [118–120].

5.3. Exosome located circRNAs

We have previously identified extracellular vesicle (EV)-packaged lncRNA HISLA as a signal transducer between immune cells and tumor cells to promote aerobic glycolysis in breast cancer, suggesting that exosome-located noncoding RNAs can be novel regulatory substances to communicate metabolic information between organs in disease [121]. Li et al. found that circRNAs are enriched in exosomes in the serum of colon cancer patients, which may serve as potential exosome-based cancer biomarkers [109]. Another study showed that circ-IARS expression was upregulated in plasma exosomes which could enter vascular endothelial cells and promote tumor invasion and metastasis [122]. Moreover, a recent study found that transferring exosomal circRNA-100338 derived from hepatocellular carcinoma (HCC) cells to vascular endothelial cells enhanced angiogenesis and promoted tumor metastasis [123]. In addition, exosomal circRNAs secreted from adipocytes can be taken in by HCC cells and sponge miR-34a to activate the deubiquitination-related singling pathway linked to the poor prognosis of HCC [110]. These studies reveal that exosome-located circRNAs are stable in peripheral circulation and function powerfully in tumor progression, reminding us that they are potential biomarkers of cancers.

Although more and more circRNAs in different subcellular fractions have been identified [124], targeting strategies for them are still limited. For example, due to the existence of the mitochondrial bilayer membrane, the efficient delivery system for mitochondrial circular RNA was lacking until the recent application of mitochondria-targeting nanoparticle (mito-NP) [13]. Moreover, the relationship between circRNA's subcellular localization and disease metabolism is still a mystery, which requires further investigation and the development of new technologies.

6. Emerging therapeutic strategies to target circRNAs

Previously, the most commonly used strategies to target circRNAs were knockdown via RNAi and overexpression via plasmids. To specifically knock down or overexpress circRNAs instead of their linear counterparts, the design of objective nucleotide sequences targeting circRNAs is quite different from conventional RNAs. For short interfering RNA (siRNA) or short hairpin RNA (shRNA), the nucleotides should be complementary to the unique back-splicing junctions of circRNAs [20,55]. The CRISPR/Cas9 system containing guide RNAs (gRNAs) targeting repeat elements or exons in the parental gene locus has recently been used to achieve circRNA knockout (Fig. 2) [19,45,57,125]. A latest study used the CRISPR/Cas13 system with gRNAs targeting back-splicing junction sites of circRNAs to achieve circRNA knockdown with greater specificity and lower mismatch tolerance (Fig. 2) [126]. To overexpress circRNAs, the pairing of intronic complementary sequences flanking the circularized exons should be included in expression vectors [9]. There are also commercial plasmids containing circRNA expressing frames which

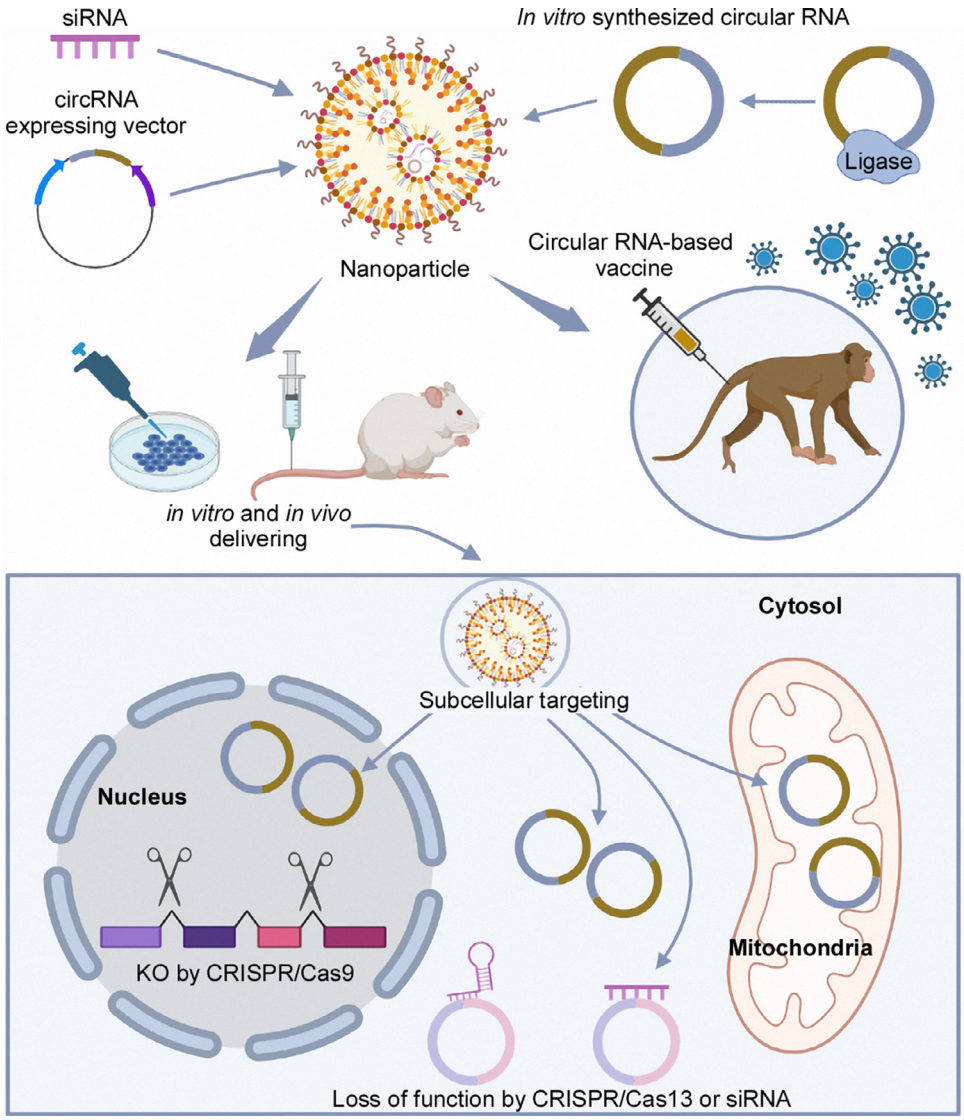

Fig. 2. Novel therapeutic strategies to target circular RNAs. Nanoparticle-delivering systems have recently been used to improve the cellular and subcellular targeting specificity of circRNAs or siRNAs. Recently, lipid-nano particles encapsulating synthesized circular RNAs have been used for *in vivo* administration to produce circular RNA-based vaccines. Another targeting strategy is CRISPR/Cas9-mediated endogenous circRNA knockout via cleaves of intronic complementary sequences flanking exons forming circRNA. In addition, circRNA could also be knockdown directly by CRISPR/Cas13 targeting the back-splice junction. Made by BioRender (https://biorender.com).

can circularize inserted sequences to form circRNAs when transfected in cells [13,58,127]. Lentivirus or adenoviral vectors are used to construct circRNA overexpressing cell lines or mouse models [128,129]. In addition, the *in vitro* synthesis of circular RNAs is a powerful strategy to directly overexpress circRNAs and it is convenient for *in vitro* studies [16,61,80,130,131]. Although some of the strategies mentioned above have been successfully tested in mouse disease models [132,133], there are still challenges in their clinical applications. The most concerning obstacles are the specificity of tissue or cell targeting and the immunogenicity of synthetic circular RNAs [6].

6.1. *circRNA targeting specificity*

Common strategies to intervene circRNAs can cause off-target effects on unexpected tissues or cells. To improve the targeting specificity *in vivo*, researchers engineered nanoparticle systems that delivered encapsulated siRNAs or circRNA expression vectors to tumor tissues and subsequently induced tumor cell apoptosis and inhibited tumor progression (Fig. 2) [134–136]. Recently, taking advantage of encapsulated cationic peptides, nanoparticles have also been used to deliver nucleotides to subcellular fractions, including the nucleus and mitochondria [137–139]. In our latest study, we encapsulated circRNA expression vectors in mitochondria-targeting nanoparticles (mito-NP) and successfully expressed mitochondrial circular RNAs, which inhibited fibroblast activation in liver disease. Moreover, delivering circular RNA SCAR *in vivo* using mito-NP could alleviate high-fat diet (HFD)-induced cirrhosis and insulin resistance [13]. Nanoparticle delivery systems may have great potency in tumor therapy. A growing body of evidence demonstrates that immunosuppressive factors and metabolic stress in the tumor microenvironment result in the disruption of the mitochondrial and metabolic state of T cells, leading to the bad efficacy of CAR-T or TCR-T cell therapy [140,141]. Therefore, targeting mitochondrial nucleic acids of T cells using mio-NP will mediate metabolic reprogramming of CAR-T or TCR-T cells and enhance the effect of immune cell therapy.

6.2. *Immunogenicity of synthetic circular RNAs*

In vitro synthesized circular RNAs have immunogenicity, for foreign circular RNAs always lack m^6A modification [80]. However, a recent study showed that synthesized circular RNA by T4 RNA ligase exhibits

little immunogenicity compared to circles produced by the group I introns with extra fragments to induce immune responses [130], suggesting that this kind of synthesized circular RNA has the potency to be applied to *in vivo* treatment without unwanted immune system activation. On the other hand, another recent study took advantage of the immunogenicity of synthesized circular RNAs to develop circular RNA-based vaccines, which could stably elicit potent neutralizing antibodies and T-cell responses to protect the host (Fig. 2) [142]. This exciting study makes circular RNA-based vaccines a potential candidate to fight against virus infection, such as the current Coronavirus disease 2019 (COVID-19). Nevertheless, the main challenge of this strategy is the artifact of circRNA impurity contributed mainly by contaminating linear dsRNA [143], although early papers by Y Grace Chen and Howard Y Chang suggest that circRNA is more immunogenic than linear mRNA [80,81]. Since the immunogenicity of circRNAs is currently under intensive study, purifying the circular form and minimizing linear RNA contamination is the major task for its clinical applications.

7. Conclusion and perspective

The regulation of circRNA expression from biogenesis to degradation is closely related to cellular homeostasis. Recent studies have uncovered circRNAs as a hallmark of various immune metabolic diseases and cancers [3,144]. Currently, there are still some unsolved open questions during the long-lasting investigation of circRNAs. Firstly, we have identified several overlong circular RNAs encoded by the mitochondria genome, including hsa_circ_0089761 with 8302 nt and hsa_circ_0089763 with 5783 nt [13]. These overlong circular RNAs pose great challenges to the study of their biogenesis and functions in diseases as well as targeting strategies. Advanced technologies are needed, such as nanopore sequencing, which can identify the full sequences of these unusual circular RNAs [32,34]; and the CRISPR/Cas13 system, which can better distinguish circRNAs from cognate mRNAs with overlapping exons [126]. Secondly, circular RNAs associated with cellular metabolism and diseases are identified in subcellular organelles, including those within the mitochondria that are produced from the mitochondrial genome and those made in the nuclear genome and translocated to mitochondria for functions [13,15,108,145]. It remains to define, however, to what extent circular RNAs are expressed in a subcellular organelle-specific manner. For example, can circular RNAs be located in the Golgi apparatus or endoplasmic reticulum? Are these special locations regulated by factors involved in immune metabolic diseases or tumor progression? Moreover, although we found that nuclei-encoded spliceosomes can regulate the generation of mitochondrial circular RNAs, how mtDNA is processed into circular RNAs after being transcribed as a large polycistronic precursor remains a mystery. Finally, since the mitochondrion has its own genome and protein translation system [146], it is of interest to investigate whether mitochondrial circular RNAs could use mitochondrial ribosomes to produce peptides. Answering these questions will further expand the regulatory mechanisms and cellular functions of circRNAs, providing new principles for disease treatment.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work.

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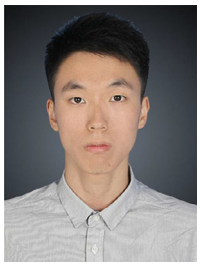

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