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REVIEW

In vitro transcribed circRNA as a therapeutic agent for cancer

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Abstract RNA therapy represents an innovative approach for cancer treatment, with several RNA-based therapeutics having received approval from the US Food and Drug Administration. Circular RNA (circRNA), a closed-loop RNA molecule characterized by its high stability, plays a significant role in regulating biological processes by modulating gene expression and facilitating protein translation. Given its unique structure and diverse functionalities, the delivery of exogenous circRNA has emerged as a novel strategy for cancer therapy. This review examines the mechanism underlying circRNA-mediated tumor therapy, emphasizing its various biological roles, including that of an RNA sponge, aptamer, gene editing tool, and facilitator of protein translation, and explores the therapeutic potential in oncology. The review provides a comprehensive discussion on the synthesis strategies of exogenous circRNA, based on T4 DNA ligase and the permuted introns-exons (PIE) method, as well as elucidating the purification techniques. This article also reviews prominent carriers currently employed for circRNA delivery, such as lipid nanoparticle (LNP), exosomes, and virus-like particle (VLP), with a particular emphasis on their application in cancer-targeted therapies. Finally, the review summarizes key challenges currently in the field along with viable solutions. It highlights the prospective role of artificial intelligence in enhancing circRNA delivery to facilitate precise cancer treatment based on exogenous circRNA.

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1. Introduction

Cancer treatment remains a central focus in modern medicine, with substantial progress made through innovative methods such as surgical resection, chemotherapy, targeted therapy, and immunotherapy. However, these approaches are still hindered by substantial side effects, narrow indications, and the development of drug resistance[1-3]. This has driven the exploration for more novel and promising therapeutic strategies. As research advances, it has become clear that RNA plays a pivotal role in biological processes. For example, leveraging the characteristics of mRNA can directly encode specific proteins in cells, and researchers have begun to deliver exogenous mRNA *in vivo* with two primary purposes. First, it can compensate for or replace the loss of protein function due to genetic defects, thereby restoring or improving normal cellular functions. Second, it can encode specific antigens to elicit targeted immune responses[4,5]. In addition to mRNA and tRNA involved in translating genetic information, numerous non-coding RNAs, such as long non-coding RNA, microRNA, circular RNA (circRNA), etc., can participate in epigenetic modification, post-transcriptional regulation, as well as regulating protein function through interactions with DNA, RNA, and proteins. By regulating the activity of these RNAs, researchers can profoundly impact the progression of various diseases[6-9].

RNA therapy primarily focuses on the development of antisense oligonucleotides, mRNA, small interfering RNA (siRNA), aptamers, etc.[10-13]. Unlike traditional pharmaceuticals that require binding to specific pockets, RNA offers the significant advantage of targeting almost all metabolic pathways within cells[14,15]. Therefore, processes in pathogenic pathways such as protein synthesis, epigenetic modification, and transport expression can serve as potential therapeutic targets for RNA therapy. Additionally, non-coding RNA involved in disease progression may also be targeted for inhibition or elimination[16,17]. Another notable advantage of RNA therapy lies in its rapid and modular development process facilitated by the flexibility of RNA sequences. Once a target RNA sequence is identified, it can be swiftly synthesized for experimentation, thereby accelerating the research and development process[18]. The stability and binding affinity of RNA can be easily improved through various chemical modifications, thereby reducing drug modification costs. Furthermore, unlike exogenous DNA, which poses the risk of integrating into host DNA, RNA remains independent of host genome integration, which greatly reduces genetic risks and improves treatment safety (Fig. 1)[19]. The unique therapeutic mechanism has generated increasing interest in developing RNA-based tumor treatments. Although many RNA therapeutics are undergoing clinical trials, challenges remain. RNA molecules are still prone to instability and degradation by nucleases. RNA can easily provoke host immune responses due to immunogenicity that not only compromises drug delivery efficiency, but also increases production cost along with storage and transportation expenses[20]. While strategies such as chemical modification and carrier-based delivery to prolong the drug's circulation time, identifying more stable RNA forms to mitigate the risk of side effects remains a key priority.

circRNA is an endogenous closed circular RNA molecule formed by reverse splicing of linear precursor RNA. Known for its high stability, circRNA can be generated through splicing at various gene sites, resulting in different circRNA isoforms. Therefore, its abundance in cells is far more than that of upstream protein-coding genes[21,22]. The abundance of circRNA is tissue-specific and influenced by physiological conditions. Although it is less abundant in most cell tissues (except the nervous system), numerous studies have confirmed that differential expression of circRNA is often associated with disease progression, especially in cancer. Increasing evidences suggest that circRNAs are

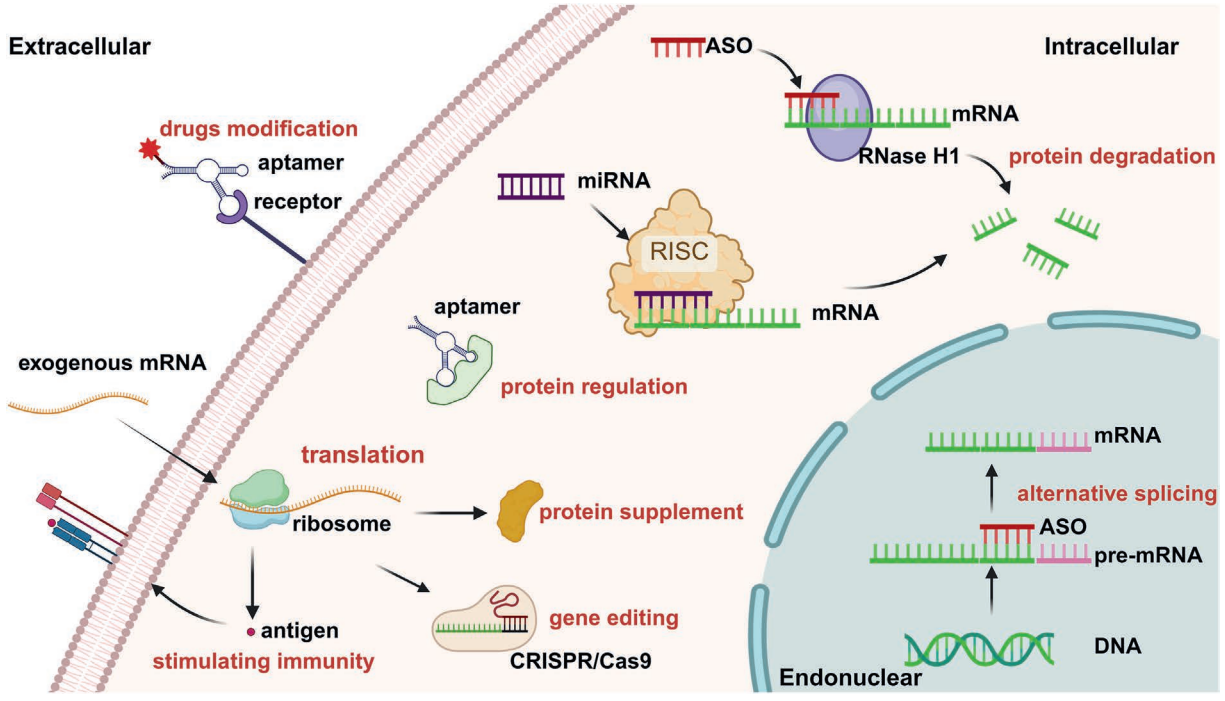

Figure 1 Overview of RNA therapy: This section covers various aspects of RNA therapy, including miRNA sponges, aptamers, protein translation mediated by the RISC complex, and gene editing techniques. Created in BioRender.com. ASO, antisense oligonucleotide; RISC, RNA-induced silencing complex.

involved in regulating tumor progression[23-26]. Functionally, circRNA can modulate the transcription and translation of target genes by interacting with miRNAs, proteins, etc. Some circRNAs also act as translation templates *via* internal ribosome entry site (IRES) or m^6A-mediated cap-dependent extracellular initiation mechanisms to produce functional peptides or proteins[27]. Numerous circRNAs have been identified as key regulators of disease progression, positioning them as potential therapeutic targets. Furthermore, variations in circRNA expression levels may also serve as valuable diagnostic and prognostic biomarkers. Based on these characteristics, the delivery of exogenous circRNA has emerged as a novel therapeutic method. The advancement of various biosynthetic technologies has facilitated the *in vitro* synthesis and circularization of circRNA. This method allows for the delivery of tumor suppressor circRNA to rectify endogenous deficiencies or leverage the high stability and persistent translation ability of circRNA to mimic the mRNA vaccines to prolong sustained protein expression and antigen delivery. With advancements in RNA biological synthesis and nano-delivery systems, circRNA is expected to play an increasingly prominent role in precision oncology treatment.

In this review, we highlight the unique physiological functions of circRNA, encompassing its role in gene expression regulation, miRNA sponge, protein interaction, and translation. We provide an in-depth exploration of its biological basis and significant potential in tumor therapy. Given the remarkable stability and low immunogenicity of natural circRNA, we further review the current methodologies for artificially synthesizing circRNA, such as *in vitro* transcription, self-splicing system, and enzymatic ligation. We expand on delivery strategies tailored for circRNA, such as lipid nanoparticle (LNP), exosome, and ligand-modified targeting systems to improve its stability and targeting efficacy *in vivo*. We also briefly discuss the application of artificial intelligence (AI) in the field of circRNA. In summary, our goal is to furnish a theoretical framework and practical insights into the translational application in tumor treatment by fully understanding the key issues and solutions in basic research and clinical development. This endeavor seeks to facilitate the early clinical implementation of circRNA therapy.

2. circRNA biological function and preparation

circRNA has garnered increasing attention due to its unique circular structure. circRNA is a highly conserved and stable covalent circular RNA molecule that connects the upstream 3′-splice junction with the downstream 5′-splice junction of RNA through reverse splicing. Once thought to be a mere byproduct of biological errors, it is now recognized that circRNA plays a crucial regulatory role in cellular processes and disease development[28-30]. The most notable characteristic of circRNA is the high stability brought by its circular structure[31]. Unlike traditional linear RNA, which is degraded through the loss of the 3′-polyA tail or *via* 5′−3′ exonucleolytic degradation after cap removal, circRNA lacks a polynucleotide tail and 5′- and 3′- ends, making it exonuclease-resistant with better stability and half-life[32]. In addition, circRNA lacks the recognition sequence for immune receptors such as retinoic acid-inducible gene I (RIGI) and Toll-like receptors, contributing to its immune evasion capabilities and allowing it to remain stable within the host for extended periods[33]. CircRNAs can be divided into several types based on their origin, including exonic circRNA, intronic circRNA, exon-intronic circRNA, intergenic circRNAs, and fusion circRNA. Exonic circRNA is the most numerous and is located in the cytoplasm, while intronic circRNA is more likely found in the nucleus[34,35].

The production of circRNAs is governed by stringent systemic regulation. Most eukaryotic circRNAs are formed by reverse splicing, the 5′- end of the downstream exon of RNA folds back in proximity to the 3′- end of the upstream exon, establishing a connection, which is also known as intron pairing-driven circularization. The inverted Alu repeat elements or complementary sequences of the introns flanking the circularized exon bring the splicing sites on both sides into proximity, form a lasso through direct base pairing, and ultimately generate circRNA following the removal of introns[36]. Another method for circRNA formation is exon skipping. In this process, RNA forms a structure near an exon and connects the downstream splicing donor with the upstream splicing acceptor through 3′ and 5′ phosphodiester bonds to form a lasso structure. Secondary splicing or intron splicing then occurs, followed by intron excision, ultimately resulting in an exon-containing circRNA[37]. The generation of intronic circRNA involves the intron 5′ splicing site is connected to the branch point to create a lasso. The 11-nt cytosine-rich branch point and the 7-nt GU-rich 5′ splicing site can protect the lasso from debranching enzymes and stabilize the loop and enable its physiological functions[38]. Since a precursor RNA may contain multiple introns, different 5′ and 3′ splicing sites can undergo different reverse splicing, ultimately leading to the formation of different circRNAs[39]. Meanwhile, various RNA-binding proteins (RBPs) can interact with distinct RNA sequences, thereby either promoting or inhibiting the circularization process[40]. This interaction plays a crucial role in regulating the differential expression of circRNA.

CircRNA was first identified in the genome of plant viroids in 1976 and initially regarded as a byproduct of RNA splicing errors. Since then, although a large number of circRNAs have been identified in the human genome, in-depth research into the mechanism has greatly benefited from the advancement of high-throughput sequencing, microarray screening, and bioinformatics algorithms[38,41]. To date, more than 100,000 circRNAs have been identified, surpassing the number of human coding genes[42]. Most circRNAs exhibit low expression levels in most tissues and are initially considered non-functional fragments resulting from mRNA mis-splicing. However, subsequent research revealed no significant correlation between the expression level of circRNA and its corresponding linear mRNA. Instead, circRNAs displayed a unique tissue- and developmental stage-specific expression pattern, suggesting that they may represent biologically active products arising from specific pathways[43]. Increasing evidence now indicates that circRNAs play crucial roles in various physiological processes and disease progression. In oncology specifically, hundreds of circRNAs have been dysregulated across different cancer types, indicating their integral role in cancer pathogenesis[42-44]. While the vast majority of circRNAs are classified as non-coding RNA, a small subset has been shown to possess protein-coding potential through cap-independent mechanisms, contributing to tumor progression.

2.1. *Biological functions of circRNA*

2.1.1. *Sponge effect of miRNA*

miRNA is a type of endogenous non-coding RNA with a length of about 20−25 nt, which can regulate gene expression by

specifically binding to the 3′ untranslated region (3′ UTR) of the target mRNA. In 2013, Hansen et al.[45,46] first reported the molecular sponge function of circRNA, revealing that ciRS-7 (circRNA sponge for miR-7) effectively adsorbs miR-7 *via* more than 70 conserved binding sites. Concurrently, Memczak et al.[47] identified thousands of circRNAs throughout the entire genome and elucidated the interaction mechanism between CDR1as and miR-7. Given that miR-7 serves as a crucial tumor suppressor capable of regulating multiple oncogenic pathways, Pan et al.[48] demonstrated that ciRS-7 significantly upregulates oncogene expression and promotes malignant tumor progression by inhibiting miR-7 activity. Conversely, deletion of ciRS-7 induces apoptosis and suppresses malignant phenotypes in tumor cells. Subsequent studies have confirmed that the molecular sponge effect mediated by circRNA is widely involved in various pathological processes such as cell cycle regulation, programmed cell death, and angiogenesis[49]. The regulatory mechanisms through which circRNA influences miRNA are diverse. For example, Hanse et al.[50] found that when ciRS-7 binds to miR-671, AGO2-dependent cleavage occurs, thereby releasing stored miR-7. Han et al. identified circLONP2 as an RNA that promotes colorectal cancer (CRC) invasion, which regulates the intercellular transmission of miRNA by directly binding to pir-miR-17 to promote maturation and subsequent exosome packaging[51]. These phenomena suggest that circRNA may function as dynamic regulatory elements capable of regulating the spatiotemporal distribution of miRNA in the local microenvironment by establishing a miRNA reservoir. This discovery provides a new mechanistic dimension for the regulatory network involving circRNA.

2.1.2. Aptamer

The biological functions of circRNA extend far beyond the classic miRNA sponge effect, emerging as a significant focus in epigenetic regulation through direct interactions with proteins that modulate their activity[52]. This interaction exhibits notable tissue specificity, with functional outcomes varying fundamentally depending on the cell type and pathological state. For example, in normal cells, circFOXO3 functions as a protein scaffold to recruit CDK2 and P21 to form a ternary complex, which halts the cell cycle at the G1/S checkpoint by inhibiting the kinase activity of CDK2, thereby terminating cell proliferation[53]. However, in malignant tumors, circFOXO3 exhibits dual regulatory properties. In breast cancer, circFOXO3 has been shown to bind to TP53 and the E3 ubiquitin ligase MDM2, facilitating the ubiquitination and subsequent degradation of mutant TP53. circFOXO3 can also inhibit the activity of histone methyltransferase WHSC1, thereby inhibiting tumor progression[54,55]. In CRC, circHAS2 influences the bidirectional regulation of CCNE2 through the miR-1244/CCNE2 pathway and competes with USP10 for binding to TP53, thereby promoting CRC cell proliferation[56]. Such functional plasticity indicates that the regulatory network governed by circRNA is highly sensitive to the microenvironment. In the realm of protein ubiquitination regulation, circRNA shows unique mechanistic diversity. For example, circRNA-CREIT specifically mediates the K48 ubiquitination degradation of PKR protein by exposing the E3 ligase active domain of HACE1, thereby inhibiting chemotherapy resistance driven by EIF2S1 phosphorylation[57]. In pancreatic cancer, CircSTX6 disrupts the assembly of the VHL−EloBC−CUL2−Rbx1 complex by competitively binding to CUL2, blocking the K63 ubiquitination degradation of HIF1A, and ultimately activating the hypoxia-induced tumor metastasis program[58]. Such studies reveal that circRNAs can influence substrate specificity by precisely modulating the spatial conformation of the E3 ligase complex. Moreover, nuclear-localized circRNAs reshape the epigenetic landscape by regulating the transcriptional machinery. For instance, EciRNA has been shown to bind U1 snRNP and further interact with RNA polymerase II at the promoter region, thus regulating parental gene expression. This mode of subcellular compartmentalized regulation provides a novel theoretical framework for post-transcriptional modifications mediated by circRNA[35].

2.1.3. Translation

Studies have shown that the 5′ UTR of the picornaviral genomic RNA contains an IRES capable of recruiting 40S ribosomal subunit to initiate translation independently of the canonical 5′ cap structure[27]. The embedded IRES with recombinant circRNA endows it with cap-independent translational capacity in the cytoplasm. Legnini et al. [59] reported that the 5′-UTR of circZNF609 functions as an active IRES, enabling splicing-dependent protein translation. High-throughput sequencing further reported approximately 17,000 sequences with putative IRES activity in circRNA and revealed that the stem-loop structure located about 40−60 nt downstream of its first nucleotide, along with an 18S rRNA complementary sequence, promotes the translation of circRNA[60,61]. In addition, expanded repeat-containing circRNA can drive non-canonical translation. An intron of the GGGGCC repeat sequence located within the *C9ORF72* gene can be circularized, and the translation of this repeat sequence generates toxic dipeptide repeat proteins[62]. Modifications such as m^6A, exon junction complex (EJC), etc., have also been shown to participate in ribosome recruitment for translation. m^6A, one of the most prevalent RNA modifications in eukaryotes, primarily targets the RRm6ACH motif, thereby influencing mRNA localization, splicing, and translation. Previous studies have demonstrated that m^6A interacts with YTHDF within the YTH domain to enhance translation efficiency. Yang et al.[63] further elucidated that a single m^6A residue can function as a ribosome recruitment site by recruiting the initiation factor EIF4G2 under the mediation of YTHDF3, which is sufficient to initiate translation in circRNA. The co-expression of m^6A methyltransferases METTL3 and METTL14 significantly augmented protein translation. Additionally, the translation of m^6A-containing circRNA is further upregulated following heat shock stress. Notably, translation of m^6A-modified RNAs has been identified in circRNAs such as circSTX6[64]. Notably, circRNA with multiple start codons can initiate translation from different sites, thereby producing multiple protein variants. circRNA can also facilitate repeated translation of the same sequence through rolling circle amplification/translation (RCA/RCT) following a single initiation event. By eliminating all stop codons from the reading frame, an infinite development reading frame is generated, substantially increasing the yield of translation products[65,66]. However, this translation method also has an endpoint. Wang et al. proved that the translation process can be halted by generating non-frame stop codons through the -1PRF mechanism[67].

In recent years, accumulating evidence has shown that a subset of circRNAs can undergo IRES-mediated translation to produce bioactive peptides that substantially influence tumor occurrence and progression. Song et al.[68] identified a highly expressed circZKSCAN1 in hepatocellular carcinoma, which has no miRNA sponge activity but encodes a functional peptide circZKSaa. CircZKSaa can interact with FBXW7 to promote mTOR ubiquitination, ultimately inhibiting cancer cell proliferation.

Similarly, circFAM53B, identified by sequencing of tissues from six breast cancer patients, has been shown to synthesize cryptic antigenic peptides in an IRES-dependent manner, significantly enhancing the infiltration of tumor antigen-specific cytotoxic T cells, thereby eliciting potent antitumor immunologic reaction[69]. These findings underscore that circRNA-encoded peptides play active roles within the tumor microenvironment and highlight new molecular targets and conceptual frameworks for circRNA-based therapeutic development.

CircRNA plays a multifaceted regulatory role in tumorigenesis and development, with its biogenesis and activity precisely modulated by multiple molecular mechanisms. These include circRNA cyclization-dependent RBPs such as Quaking, DHX9, as well as transcriptional regulation and epigenetic modifications[40]. Beyond direct strategies such as siRNA-mediated knockdown and gene editing, numerous exogenous small molecules—including natural compounds like curcumin, matrine, and berberine that have been shown to modulate circRNA abundance by targeting RBPs, spliceosome components, or RNA modifications[70]. These components also regulate oxidative stress homeostasis to reduce circRNA synthesis. Furthermore, many therapeutics have been demonstrated to interfere with the circRNA metabolic axis. For instance, metformin combined with radiotherapy suppresses TR4 expression, thereby affecting the Quaking/circZEB1/miR-141-3p/ZEB1 regulatory cascade and inhibiting prostate cancer progression[71]. Notably, several small molecules targeting RNA modification or RBPs have progressed into clinical trials. One representative example is STC-15, which directly targets METTL3 (involved in m^6A modification) and has exhibited promising anti-tumor activity across various solid tumor models[72]. Although such agents do not directly regulate circRNAs themselves, they reshape the synthesis and functional pathways by influencing RNA modification processes and RBP dynamics. Looking ahead, continued progress in small-molecule screening and RNA-targeted drug discovery target RNA is expected to facilitate the clinical translation of therapeutics that modulate circRNA biology[73].

To address the demands of large-scale clinical applications, researchers have accelerated the optimization of *in vitro* circRNA synthesis and purification workflows to obtain high-purity, biologically active products. Advances in multiple technologies have enabled the successful construction of circRNA using diverse biochemical and enzymatic methods. Coupled with the development of circRNA delivery systems-such as LNP, VLPs, or exosomes, makes it possible to apply highly stable, efficiently expressed circRNA in precision tumor treatment and vaccine development.

2.2. *Preparation and purification of circRNA*

2.2.1. *Preparation of circRNA based on the T4 ligase ligation method*

Generally, the *in vitro* synthesis of circRNA consists of two primary steps: synthesis of linear precursor RNA and cyclization (Fig. 2)[74]. Linear precursor RNA can be obtained either chemically or enzymatically[75,76]. At present, chemical synthesis of linear RNA predominantly relies on amido phosphorus chemistry and solid-phase synthesis. Its major advantage is the precise control over protecting-group removal. Its major advantage is the precise control over protecting-group removal and 3′−5′ phosphordiester bond formation, which minimizes undesired 2′−5′ linkages and other side reactions, enabling the synthesis of high-fidelity short RNAs (usually≤70 nt). However, this method is limited by high purification costs and inherently low yields, rendering it unsuitable for producing long-chain RNA. In contrast, the enzymatic strategy employs bacteriophage RNA polymerase (such as T7 RNA polymerase) for *in vitro* transcription. This approach supports efficient synthesis of long RNA precursors up to several thousand nucleotides in length. Following precursor synthesis, it is essential to ligate its head and tail to form a stable, closed circular structure. Currently, cyclization strategies primarily include chemical and enzymatic ligation. Chemical ligation typically relies on the condensation reaction between the 5′-phosphate and 3′-hydroxyl groups of linear RNA, employing reagents such as cyanogen bromide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and azide-alkyne cycloadditions. However, this approach generally exhibits low ligation efficiency and frequently generates non-natural 2′−5′ phosphate bonds, limiting their suitability for high-fidelity circRNA production[77]. Consequently, the milder enzymatic ligation method has emerged as the preferred strategy. The enzymatic strategies, such as T4 ligase ligation and ribozyme ligation, are considered the mainstream approaches for *in vitro* synthesis of circRNA. T4 DNA ligase (T4 Dnl), T4 RNA ligase 1 (T4 Rnl 1), and T4 RNA ligase 2 (T4 Rnl 2) are widely utilized for *in vitro* cyclization. A prerequisite for enzymatic cyclization is that the acceptor region carries a 3′-OH and the donor region contains a 5′-phosphate. This configuration ensures ligation specificity and substantially improves the cyclization efficiency and product purity[78].

T4 Dnl catalyzes phosphodiester bond formation at the break of DNA−DNA or DNA−RNA heterochain regions, thereby circularizing linear precursors through the "splint" strategy. Specifically, a short complementary DNA oligonucleotide matches the sequences flanking the ligation junction, effectively serving as a bridging scaffold. Its ends respectively bind to the 5′-phosphate and 3′-hydroxyl of the RNA precursor to form a stable hybrid region recognized by T4 Dnl. Upon ligation, the enzyme seals the nick to generate a covalently closed circRNA and is then released.

Compared with single-stranded RNA, double-stranded dsDNA and DNA−RNA hybrid regions offer higher substrate affinity for T4 Dnl, resulting in greater efficiency for dsDNA ligation than DNA-RNA hybrids. The high selectivity of T4 Dnl contributes to improving the accuracy during circularization. However, the T4 Dnl method is still subject to several conditions in circRNA synthesis. The RNA precursor cannot form a stable secondary structure at the ligation junction; otherwise, it will hinder effective annealing of the cDNA splint and formation of the duplex. An excessive number of uracil (U) residues diminishes the thermal stability of the duplex, thereby affecting the catalytic activity of T4 Dnl[79]. Kim et al.[80] designed a Gap-DNA splint incorporating additional nucleotides that accurately matched the gap position, enabling T4 Dnl to leverage the surrounding stable duplex while ensuring spatial accessibility at the ligation site, which significantly improved the circularization efficiency. Li et al.[81] constructed a double-armed circular DNA-dumbbell splint that provides stable junction interfaces at both termini and successfully circularized circRNAs ranging from 16 to 44 nt in length.

T4 Rnl 1 catalyzes the nucleophilic attack of the 3′-OH ends on the activated 5′-end to form a 5′,3′-phosphodiester bond, thereby mediating RNA ligation and cyclization. Compared with T4 Dnl, T4 Rn1 is restricted to single-stranded RNA substrates and exhibits lower reaction specificity[82]. It also demonstrates a sequence preference at the ligation site, adenosine (A) as the 3′-end acceptor while cytidine (C) is preferred as the 5′-end

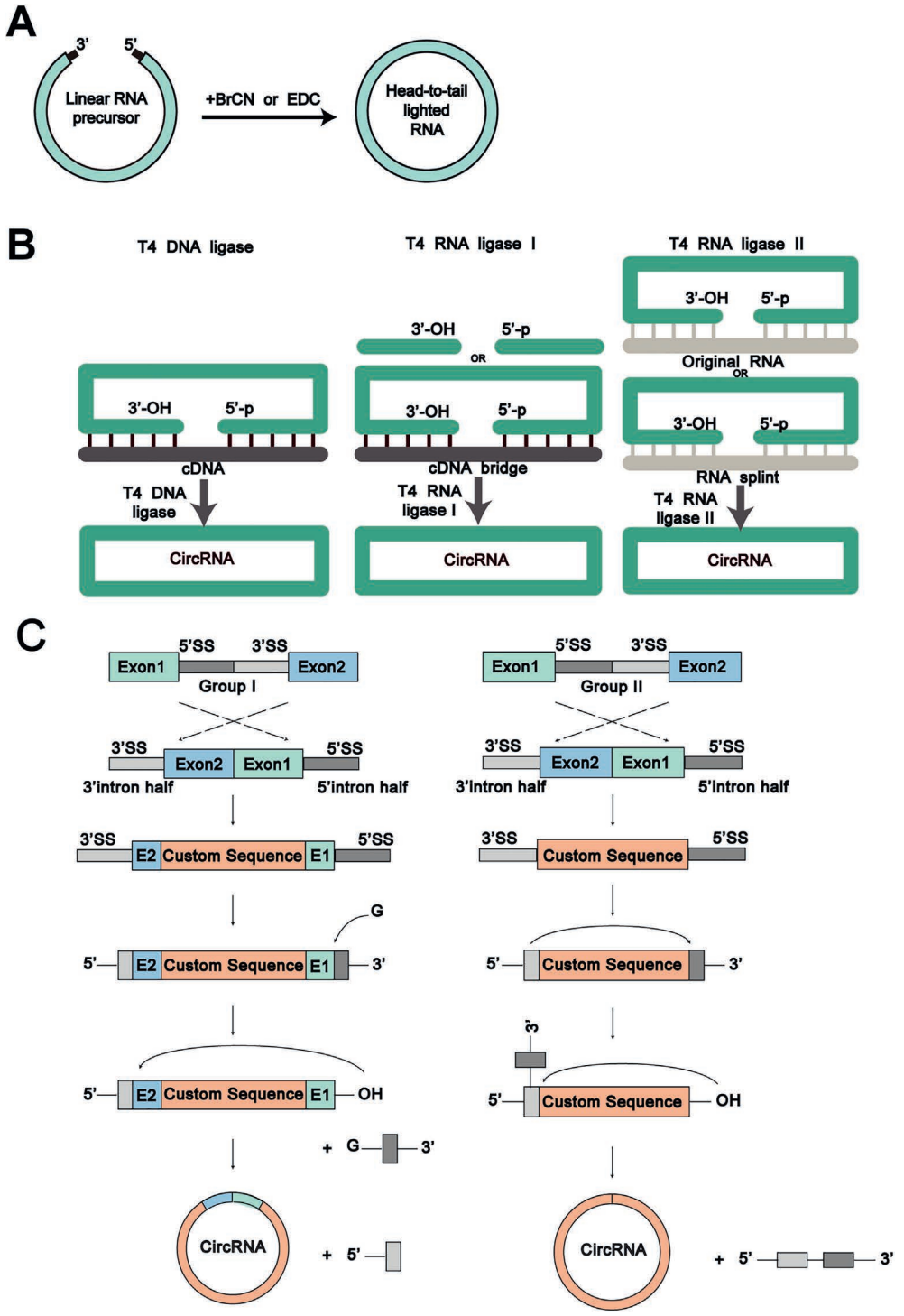

Figure 2 Methodological strategies for *in vitro* circRNA synthesis. (A) Chemical Method: employing cyanogen bromide (BrCN) or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to facilitate the condensation and circularization of linear RNA ends. (B) T4 ligase method: Using T4 DNA/RNA ligase to bring the ends to converge through cDNA bridges or RNA splints, catalyzing the circularization of RNA or DNA chains. (C) Generation of circRNA Using the PIE Method: constructing a circular structure based on either type I or type II intron self-splicing systems.

donor[83]. The ligation efficiency of T4 Rnl 1 is also greatly diminished when the termini of the linear RNA precursor form stable secondary structures. Furthermore, there is a side reaction involving intermolecular end ligation (oligomerization), which greatly limits the RNA cyclization. To enhance the efficiency of cyclization, a partially complementary nucleic acid splint can be introduced to position the reactive ends into proximity while maintaining the two or three terminal nucleotides in an unpaired state, thereby increasing the accessibility of the enzyme. In practical applications, T4 Rnl 1 has been successfully used to

synthesize therapeutic circRNAs. For example, Liu et al.[84] used T4 Rnl 1 to synthesize a circRNA sponge binding with miR-21, achieving targeted suppression of miR-21 function *in vitro* and demonstrating potential therapeutic value. In summary, although T4 Rnl 1 is enzymatically feasible in single-stranded RNA cyclization, its low specificity, high byproduct formation, and sensitivity to secondary structure still constrain its application in large-scale circRNA preparation. Incorporating nucleic acid splints significantly improves the ligation efficiency and product purity, providing a feasible pathway for downstream clinical or research applications.

T4 Rnl 2 catalyzes the formation of a covalent 3′−5′-phosphodiester bond between the 3′-OH and 5′-phosphate termini of RNA, similar to T4 Rnl 1, but with markedly higher ligation activity toward double-stranded RNA[85]. The ligation of single-stranded RNA ends can be facilitated by introducing an exogenous RNA splint, which facilitates alignment. The advantage of T4 Rnl 2 is its superior catalytic efficiency compared to other ligases; it requires only a minimal base pair at the ligation site[86]. Efficient cyclization requires that the linear RNA precursor be longer than 30 nucleotides. Additionally, high substrate concentrations with T4 Rnl 2 may lead to intermolecular aggregation, which reduces overall yield. Researchers have optimized RNA precursors' secondary structures to minimize pairing at the ligation sites. Notably, maintaining only two or three base pairs on the 3′-OH is sufficient, whereas the missing base pairs on the 5′-phosphate side do not hinder circularization and significantly suppress intermolecular connections. Consequently, effective circularization can be achieved from Rnl 2 without any splints required. Rapid base pairing accelerates intermolecular connections while preventing molecular aggregation[87]. This method demonstrates exceptional selectivity (>95%) even at high RNA concentrations (*e.g.*, up to 50 μmol/L). Polymer byproducts can be further suppressed by diluting the Rnl 2 buffer concentration. Similarly, some studies have employed computational modeling to simulate the secondary structure of the target circRNA and identify cleavage sites that promote intramolecular base pairs at termini. This strategy has also achieved efficient RNA cyclization under high concentrations of T4 Rnl 2. Nevertheless, both the yield and versatility remain constrained by the heterogeneity of RNA sequence and structure. Future strategies combining chemical modification with high-throughput screening are expected to accommodate a wider range of circRNA *in vitro* synthesis.

In general, the T4 ligase-based synthesis method presents several advantages, including mild reaction conditions, straightforward system control, and optimization of substrates through prediction software. Theoretically, it allows for controllable circularization of linear sequences without introducing impurities. Comparative studies on various RNA circularization strategies indicate that while T4 Rnl is not highly efficient for long circRNAs relative to the permuted introns-exons (PIE) method, it demonstrates superior performance with short RNAs[79]. Specifically, the resulting products introduce only 1−3 extra nucleotides, thereby exhibiting lower inherent immunogenicity (in contrast to 5%−10% observed in dephosphorylated linear RNA). Moreover, the synthesized circRNAs are capable of forming 21-bp and 32-bp short RNA duplexes that effectively inhibit PKR activation, demonstrating significantly higher efficiency than commercially available PKR inhibitors[88]. The combination of low immunogenicity and high biological activity endows T4 ligase with unique medical potential in preparing short circRNAs as aptamers, short peptide-coding sequences, and probes.

2.2.2. *Preparation of circRNA based on the PIE method*

The PIE method is a widely adopted technique for circularizing linear RNA by leveraging the catalytic properties of self-splicing introns. This *in vitro* circular RNA synthesis system is based on the self-splicing characteristics of type I introns derived from the td gene in T4 phage or tRNA Leu from Anabaena[89]. The type I intron self-splicing system requires only GTP and Mg^{2+} as cofactors. During the process, a continuous transesterification reaction occurs independently of spliceosomes or other proteins. The ten conserved helical structures formed upon folding of type I introns, designated P1−P10, constitute the core of the catalytic center, with P1 and P10 corresponding to two splice sites. Following folding, exogenous GTP initiates a nucleophilic attack on the phosphate at the 5′ site, resulting in the release of 3′-OH. The generated 3′-OH subsequently attacks the downstream 3′ splice site, forming a new phosphodiester bond connecting upstream and downstream exons while incorporating minimal intronic sequences to produce the circRNA. Initially, type I intron self-splicing was employed to synthesize short RNA sequences ranging from 58 to 124 nt *in vitro* with circularization efficiency exceeding 80%[90]. Wesselhoeft et al.[90] advanced this method by developing an engineered substitution group I PIE system, which introduced complementary homologous arms at both ends of precursor RNA to spatially bring the splice sites closer and enhance their interaction. Spacer sequences were also incorporated between the 3′ splice site and IRES, facilitating circularization of sequences up to 5 kb in length by promoting optimal secondary structure formation necessary for ribozyme activity. Qi et al.[91] refined the approach by designing combinations of P10 and P1-ex with varying structural strengths tailored to the sequences of different gene of interest (GOI) sequences, achieving efficient and precise optimization. Despite its advantages of straightforward reaction conditions and independence from exogenous proteins, residual sequences remaining on the circRNA may lead to scar formation, which may introduce sequence discrepancies or generate complementary sequences capable of forming double-stranded RNA. This could potentially alter circRNA or trigger immune responses, highlighting the need for further optimization[92]. Although the PIE method operates under relatively straightforward reaction conditions, the introduction of exogenous exon sequence may elicit an immune response, necessitating further optimization[88]. In this regard, Rausch et al.[93] designed a customized PIE transcription template wherein its 5′ and 3′ end sequences simulated the E2 and E1 fragments of the T4 td 1 group intron, thereby achieving accurate RNA circularization without introducing exogenous exon sequences. Similarly, several researchers have used Tetrahymena I group intron ribozymes to conduct end-to-end self-targeting and splicing reactions and successfully synthesized circRNA without introducing exogenous fragments[94]. These enhanced methodologies preserve the advantages of the PIE for large RNA precursors while eliminating immune risks, offering a feasible platform for large-scale and accurate circRNA production. By constructing PIE plasmids, circular expression can also be achieved within cells, further expanding its potential application.

Group II introns are self-splicing ribozymes widely distributed across the genomes of plants, lower eukaryotes, and bacteria. This system has also been utilized to generate circRNA *in vitro*, employing a mechanism analogous to that of group I introns and pre-mRNA introns[95,96]. The 5′ splice site at the exon terminus is precisely linked to the 3′ splice site at its beginning, yielding a circular RNA molecule. Unlike type I intron self-splicing systems,

group II introns derived from yeast mitochondrial genomes can circularize without incorporating native exons, enabling more precise ligation of linear RNA precursors. Most type II introns exhibit a conserved structure of six stem-loops (DI-DVI), which contain multiple exon-binding sequences capable of interacting with corresponding intron-binding sequences within the exon region[97]. The synergistic effect arising from this hinged stem-loop architecture stabilizes the reaction complex and enhances splicing efficiency. Catalysis by group II introns involves a two-step transesterification reaction that does not necessitate external GTP intervention. In this process, the 2′-OH group on the adenosine residue within the intron directly attacks the 3′ splice site of the upstream exon, forming an intermediate lasso-like structure. Subsequently, DVI rotates to position the newly formed 3′-OH from the upstream exon near the active sites, which then attacks the 5′ splice site to complete splicing[98]. Given that our understanding of group II intron cyclization mechanisms remains incomplete, further investigation is warranted to broaden their applicability for *in vitro* synthesis of circRNA using these self-splicing systems[99,100]. Compared with the conserved P1−P10 helical structure of group I introns, group II introns demonstrate a remarkable ability to rearrange the splice site without compromising the integrity of the catalytic center. This property more closely mimics the natural mechanism of eukaryotic spliceosomes. Additionally, group II introns do not necessitate exogenous GTP, elevated temperatures, or high concentrations of Mg^{2+}, simplifying the reaction process and minimizing byproduct contamination such as scar sequences. Their activity is also independent of the rigid P1−P10 secondary structures. By substituting exon-binding and intron-binding sequences while maintaining base-pairing fidelity, it is possible to achieve artificial programming that facilitates seamless and more precise splicing. Hu et al.[101] designed a PIE platform to synthesize circular mRNA (cmRNA) without an exogenous exon, and found that cmRNA-based anti-CD19 CAR-T effectively eliminated target cells *in vivo* and provided sustained anti-tumor efficacy. This study convincingly established the feasibility of such cmRNA for targeted cell elimination and long-term maintenance of anti-tumor activity *in vivo.*

Hairpin ribozyme (HPR) is an enzyme employed for circRNA synthesis *in vitro*. Originating from subviral genomes, HPR enables circRNA production *via* a rolling circle reaction combined with the self-splicing on a circular single-stranded DNA template[102]. Circular DNA undergoes rolling circle transcription catalyzed by T7 or *E. coli* RNA polymerase to produce a linear RNA concatemer, which further undergoes self-cleavage and ligation to form RNA circles. This method is suitable for efficiently producing small circRNA (about 50−150 nucleotides). However, the circRNA generated *via* HPR retains the ribozyme sequence, which may adversely affect the function or induce off-target effects. The inherent ribozyme activity can compromise structural stability[83]. While the HPR rolling circle strategy shows high efficiency for small circRNA production, further optimization through engineering or chemical passivation is necessary to preserve both functional integrity and structural stability. Ultimately, the choice of preparation method must be carefully tailored to the unique structural attributes of the circRNA to maximize yield and functional integrity (Table 1).

2.2.3. *Purification of exogenous circRNA*

In addition to circRNA, *in vitro* cyclization systems often generate impurities, including DNA templates, linear RNA, dsRNA, enzymes, and free introns. Linear RNA and other contaminants can be recognized by innate immune receptors, potentially triggering innate immune responses. While such impurities may serve as adjuvants in tumor vaccines and are beneficial, overall purity still needs to be maximized to avoid compromising circRNA function[103]. The residual introns from self-cyclization systems also interfere with structural characterization and functional evaluation. Currently, common purification methods include RNase R digestion, high-performance liquid chromatography (HPLC), and electrophoresis-gel recovery[104]. RNase R is an exonuclease that degrades RNA from the 3′−5′ direction[105]. Since circRNA lacks the exonuclease target sites, it can resist degradation, while linear RNA will be hydrolyzed. However, long-term exposure to RNase R will also cause the formation of gaps and degradation of circRNA, necessitating careful control of enzyme concentration, reaction temperature, and duration[106]. Polyacrylamide gel

Table 1 Comparison of different circRNA cyclization methods.

Method	Mechanism	Advantages	Limitations	Ref.
Chemical ligation	Condensation reaction *via* EDC, BrCN, forming 2′,5′-phosphodiester bonds	No biological components introduced	Low ligation efficiency; complex synthesis	75,77
T4 Dnl	DNA splint-guided complementary ligation	High ligation accuracy	Low efficiency; template-dependent	78,79,81
T4 Rnl 1	Nucleophilic attack of the 3′-OH ends on the activated 5′end	High efficiency; low immunogenicity	Low efficiency for long RNAs; inter-ligation; structure-dependent	83
T4 Rnl2	3′-OH and 5′-phosphate covalent linkage	Higher efficiency; precise ligation; low immunogenicity	Limited for long RNAs; end-joining artifacts	83,85,87
PIE Group I	Consecutive transesterification reactions mediated by GTP and Mg^{2+}	Simple; suitable for large RNAs; high-throughput synthesis	Leaves scar sequence; structure-sensitive	88-90
PIE Group II	Two-step transesterification reaction (GTP-independent)	Precise; scarless; mild conditions	Sequence-dependent; product heterogeneity	76,98,99
HPR	Self-folded stem-loop catalysis	Simple; good for short RNAs	Introduces HPR motif; unstable reaction	75,83,102

BrCN, cyanogen bromide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; PIE, permuted intron-exon; T4 Dnl, T4 DNA ligase; T4 Rnl, T4 RNA ligase; HPR, hairpin ribozyme.

electrophoresis and agarose gel electrophoresis separate RNAs based on molecular size and conformation *via* a sieving network under an electric field. Target circRNAs can be extracted from specific bands[96]. However, this method is only suitable for laboratory-scale trace recovery and purification, and the heat generated during electrophoresis may alter the secondary structure, thereby affecting the yield[107]. Size exclusion chromatography separates molecules based on size and polarity for separation, offering good resolution for large molecules and dsRNA, but has limited separation efficiency for circRNAs and nicked circRNAs of similar size. Therefore, combined methods are often employed. Wesselhoeft et al.[90] integrated Size exclusion chromatography with RNase R digestion to first remove an excess linear impurity RNA, then enhance the separation of circRNA from various impurities, obtaining a purity of more than 90%. Zhang et al. developed a multi-step purification strategy based on RNase R, macroporous cellulose, and phosphatase, progressively removing precursor RNA, dsRNA, and phosphate groups, attaining a purity reached 95% with a yield of more than 70%, ensuring the efficiency of circRNA translation (Fig. 3A)[108]. Ultrafiltration has also proven effective for circRNA purification by exploiting the differences in flux between circRNA and linear RNA through

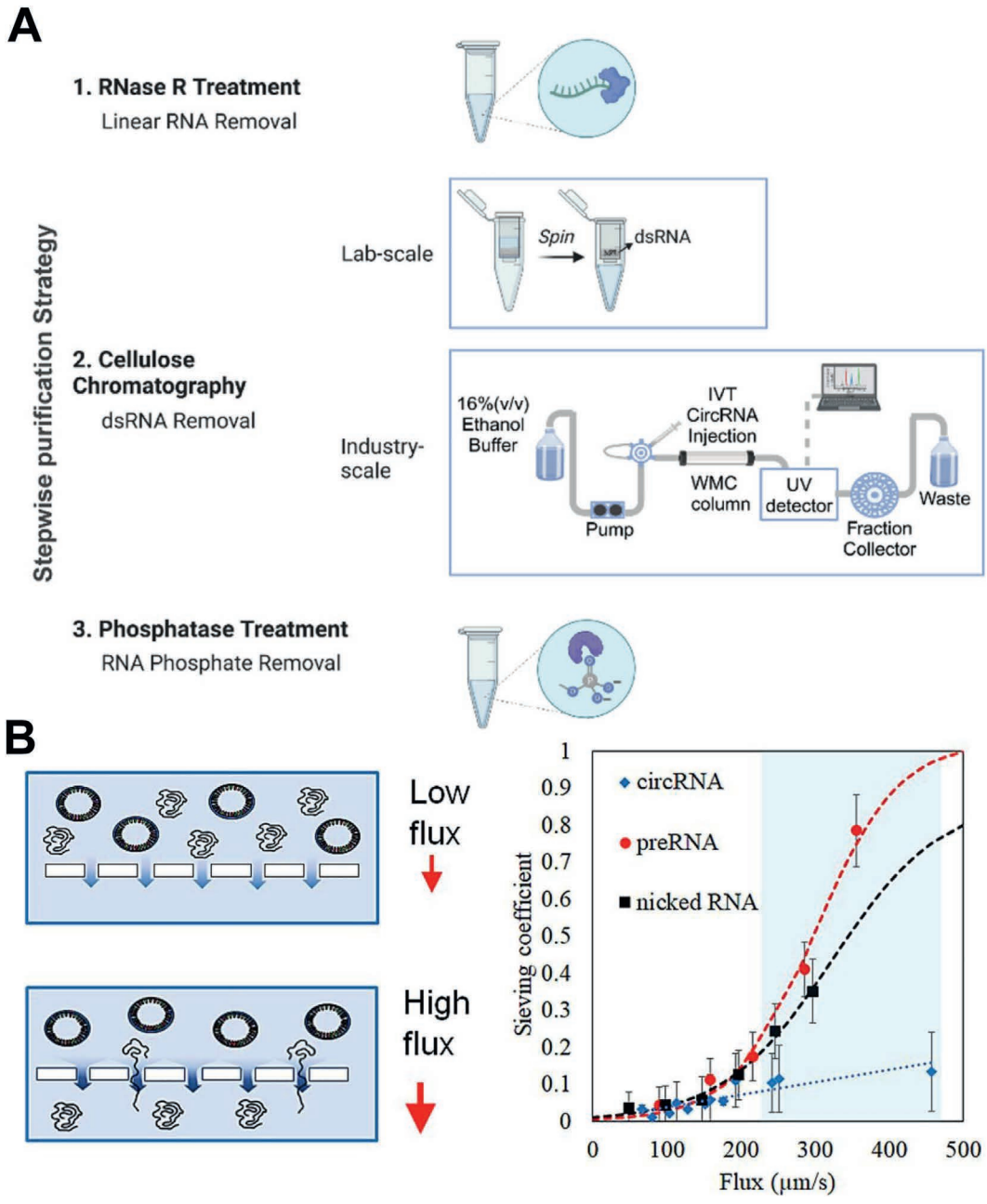

Figure 3 Strategies for the purification of *in vitro* transcribed circRNA. (A) Cellulose chromatography combined with enzymatic methods is employed to achieve a higher purity of circRNA. Adapted with permission from Ref. 108 © 2025 The Author(s). Advanced Science published by Wiley-VCH GmbH. (B) An ultrafiltration method is utilized for the purification of circRNA, adapted with permission from Ref. 109 © 2025 The Authors. Published by Elsevier B.V.

ultrafiltration membranes (Fig. 3B)[109]. Ultimately, successful purification relies on the physicochemical properties of the molecules and typically requires a combination of separation and purification to maximize purity while minimizing losses. Developing scalable purification technologies is critical for large-scale production, enabling rapid and modular production of circRNA. Thanks to the advances in these methods, a variety of exogenous circRNAs are being developed for tumor treatment. The highly stable structure allows it to efficiently exert anti-tumor effects *via* mechanisms such as RNA sponges, aptamers, or protein translation.

Synthetic circRNA maintains the low immunogenicity of natural circRNAs while enabling a broader range of functional applications through flexible sequence design. Natural circRNA exhibits a highly stable, covalently closed circular structure and is shielded from *in vivo* immune recognition by modifications such as m^6A[110]. Exogenous circRNA can reduce immunogenicity by mimicking these modifications, simultaneously enhancing stability during translation and thereby extending half-life *in vivo*. Natural circRNA possesses various physiological functions, including miRNA sponge effects and aptamer activities. Exogenous circRNA can replicate these functions through targeted functional sequence design and further optimization strategies, such as multi-site functional integration or the incorporation of efficient translation modules, to achieve specific therapeutic outcomes.

3. Application of exogenous circRNA in tumor treatment

3.1. Application of exogenous circRNA in regulating tumor RNA

The development of targeted delivery systems that harness the tumor-suppressive properties of circRNA has emerged as an important strategy to address the limitations of traditional RNA therapeutics, such as poor stability and high immunogenicity. At present, two primary approaches are employed to establish circRNA function in tumors. One is to directly deliver highly active circRNA synthesized *in vitro*, while the second entails delivering plasmids encoding circRNA *via* vectors for endogenous expression. These therapeutic circRNAs are typically further optimized through the incorporation of functional motifs or self-replicating elements to enhance anti-tumor efficacy while reducing off-target effects. Candidate circRNA is mainly identified from patient tumor tissues through high-throughput sequencing[111]. Although most identified circRNAs discovered to date promote progression, a subset exhibits tumor-suppressive functions and has achieved promising results in both *in vitro* and *in vivo* experiments. Exogenous circRNA delivery generally relies on nanocarrier encapsulation of chemically modified circRNAs or direct electroporation to achieve tumor-targeted accumulation and functional release. For example, the circRNA (hsa_circ_0009361) found by Geng's team[112] in CRC patients is used as a tumor suppressor sponge for miR-582. By blocking the inhibition of miR-582 on adenomatous polyposis coli 2, it inhibits the activity of the Wnt/CTNNB1 signaling pathway, significantly downregulates the proliferation of tumor cells, and delays the growth and metastasis of tumors *in vivo*. Such circRNAs typically exert anti-tumor effects through diverse suppressive pathways and may also be co-administered with chemotherapeutics or immunomodulators to further improve the efficacy. Another tumor-suppressive circRERE has been shown to stabilize MAVS by sponging miR-6837-3p, further activate the IFNA pathway, and promote anti-tumor immunity. Ding et al.[113] demonstrated that administration of circRERE-adeno-associated viruses (AAV) significantly reduced tumor burden. Tumors treated with circRERE-AAV expressed elevated MAVS, leading to recruitment of T cells in an IFNα-dependent manner. When combined with anti PD1 therapy, this approach achieved the highest level of T cell infiltration.

Plasmid-based circRNA overexpression relies on the rational design of linear precursors to complete self-circularization within cells. This technology traces back to the PIE system developed in the 1990s, which uses reverse-splicing elements to promote the synthesis of circRNA. Compared with *in vitro* synthetic delivery, plasmid delivery offers a lower scale production cost and greater feasibility for large-scale manufacturing. Furthermore, modular elements such as tissue-specific promoters and ribozyme self-cleavage units enable precise spatial and temporal control of circRNA expression. The shortcomings of plasmid delivery are the unwanted byproducts and the immunogenicity risk of the plasmid. Research has been conducted on encapsulating plasmids within nanocarriers for tumor treatment. For example, circUGP2, identified by Chen et al.[114], acts as a tumor suppressor factor. It regulates *ADGRB1* expression through sponge miR-3191-5p, and can also directly activate *ADGRB1* transcription to activate the tumor suppressor pathway. The researchers encapsulated the circUGP2 plasmid into LNP to achieve targeted delivery and observed a potent tumor suppressor effect (Fig. 4).

3.2. Application of exogenous circRNA in regulating tumor protein activity

Beyond acting as miRNA sponges, circRNA can also regulate protein activity with spatiotemporal precision by dynamically interacting with specific functional proteins, forming an intricate regulatory network. The researchers used the circFOXO3−p53−MDM2 axis to construct an *in vivo* circFOXO3 protein scaffold in a breast cancer mouse model. Upon binding to the histone methyltransferase WHSC1, circFOXO3 disrupts the WHSC1−H3K36me2−Zeb2 pathway and effectively suppresses tumor growth[55]. As a stable small molecule with high affinity, the development of aptamer therapeutics has become a prominent research focus in recent years to precisely target key immune regulatory factors and adhesion factors. Computational optimization, including AI-guided design, is expected to further enhance selectivity and binding efficiency. Currently, a variety of aptamer-targeted drugs are undergoing clinical trials. The cyclization of aptamers represents a promising therapeutic direction. Ring-shaped aptamers can integrate several functional motifs, enabling multi-pathway blockade within a single molecular framework. Zhou et al.[115] developed an artificial circRNA containing three elements targeting CTNNB1 and NFKB. This construct specifically inhibited dual-target signal transduction in bladder cancer cells. The team further used CD63-HuR fusion protein to facilitate exosomal loading of circRNA and blocked the activity of target transcription factors and inhibited the invasion of tumor, and outperformed conventional CRISPR-dCas9-KRAB-based strategy.

In recent years, the incorporation of IRES or m^6A-mediated cap-independent translation elements has enabled circRNA to overcome the traditional definition of non-coding RNA and continuously produce functional proteins. This feature enables circRNA to not only serve as a protein regulatory element, but also

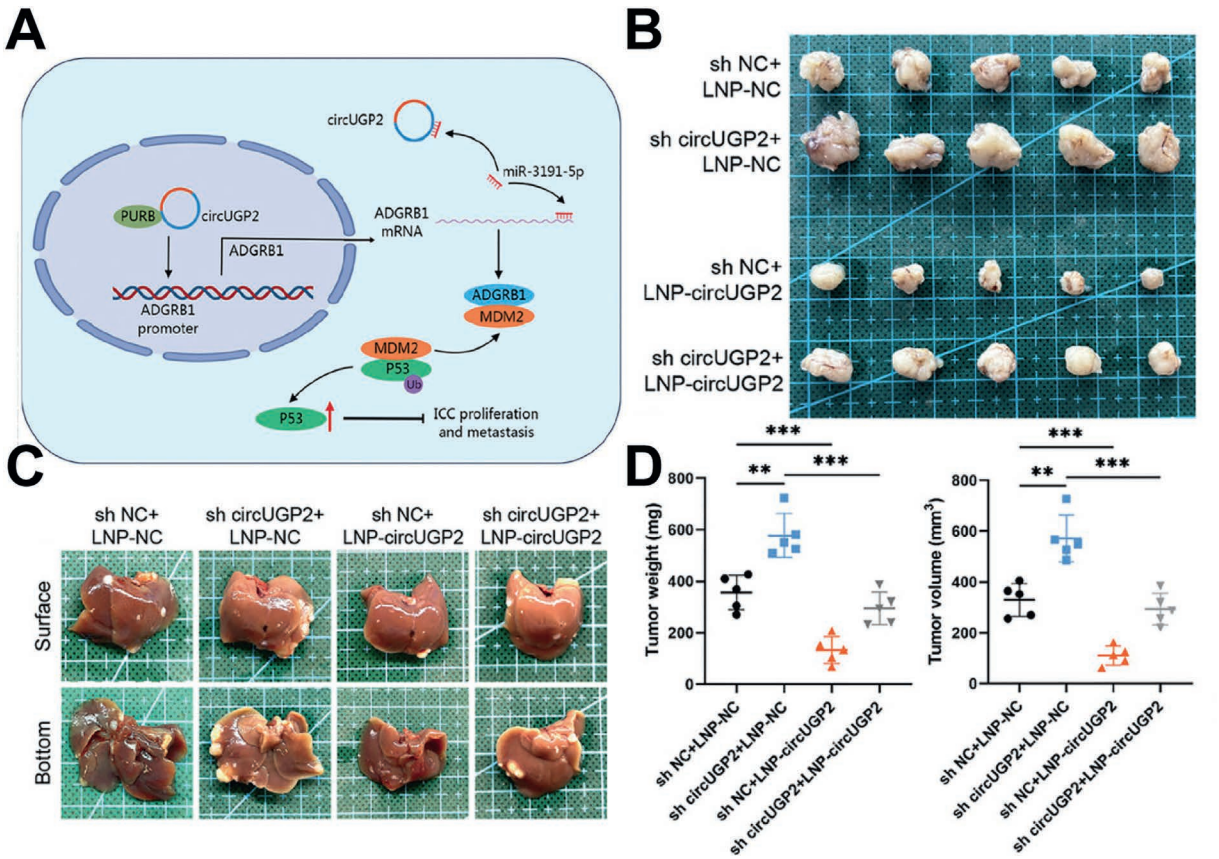

Figure 4 The miRNA sponge effect of circRNA. (A) CircUGP2 influences the activation of the ADGRB1/TP53 axis *via* sponging miR-3191-5p, thereby affecting the progression of intrahepatic cholangiocarcinoma. (B−D) The therapeutic strategy involving the delivery of circUGP2 demonstrates significant anti-tumor effects. Adapted with permission from Ref. 114 © 2024 The Author(s). Advanced Science published by Wiley-VCH GmbH.

directly serve as a protein-producing platform *in vivo*, offering unique advantages for tumor vaccine development and the delivery of gene editing tools. CircRNA can encode tumor-suppressive proteins directly. Due to their stable circular structure and reduced immunogenicity, circRNAs (median half-life: 1.8−23.7 h) typically exhibit a half-life approximately 2.5-fold longer than that of linear mRNAs (4.0−7.4 h)[31]. This extended stability results in a significantly longer translational half-life (80 *vs.* 45 h), ultimately leading to enhanced protein output over their lifespan, approximately 54.5% more than modified mRNA counterparts. The engineered circRNA translation system, Tornado, achieved an approximate tenfold increase in cellular luminescence intensity and duration of mRNA expression. Notably, the cellular luminescence intensity generated following VLP encapsulation was over fivefold greater than that observed with VLP-mRNA alone[116]. These findings underscore the substantial advantages of circRNAs in augmenting translational efficiency. With rational design of upstream sequences, it is theoretically possible to synthesize the target protein both *in vivo* and *in vitro*, including within the nucleus, cytoplasm, and the membrane. A representative example is the delivery of a circRNA adenovirus complex expressing SHPRH-146aa, which can further specifically upregulate P21 to block the cyclin/CDK pathway and induce cell cycle arrest to inhibit tumor growth (Fig. 5)[117]. The ability of circRNAs to sustain specific antigens or effector protein production makes them well-suited for cancer immunotherapy.

3.3. Application of exogenous circRNA in encoding tumor antigens

Another major therapeutic approach for exogenous circRNA is manufacturing circRNA vaccines aimed at expressing specific antigens. RNA vaccines have attracted significant attention for their ability to rapidly induce intracellular antigens. However, traditional mRNA vaccines are easily susceptible to RNase degradation during delivery, transport, and storage[118]. Although nucleotide modification can enhance the stability of mRNA, it also increases the cost and manufacturing complexity. In addition, the inherent thermal instability of mRNA necessitates low-temperature cold chain storage[119]. The primary advantage of circRNA over ordinary linear RNA lies in its enhanced stability brought by its circular structure, and the absence of pathogen recognition receptors prevents it from being cleared by the host immune system. Using *in vitro* transcription enables plasmid DNA can be converted into linear RNA precursors and then be cyclized *in vitro* to form circRNA, while translation is obtained by integrating IRES or adding m^6A modification upstream of open reading frame. Chen et al. advanced this concept by installing a branched m^7G-cap onto circRNA through a click-chemistry approach that conjugates an m^7G moiety to a predetermined azide site. This modification allows for the recognition by EIF4E and confers cap-dependent translation while preserving the stability of circRNA. As a result, this approach achieves over 100-

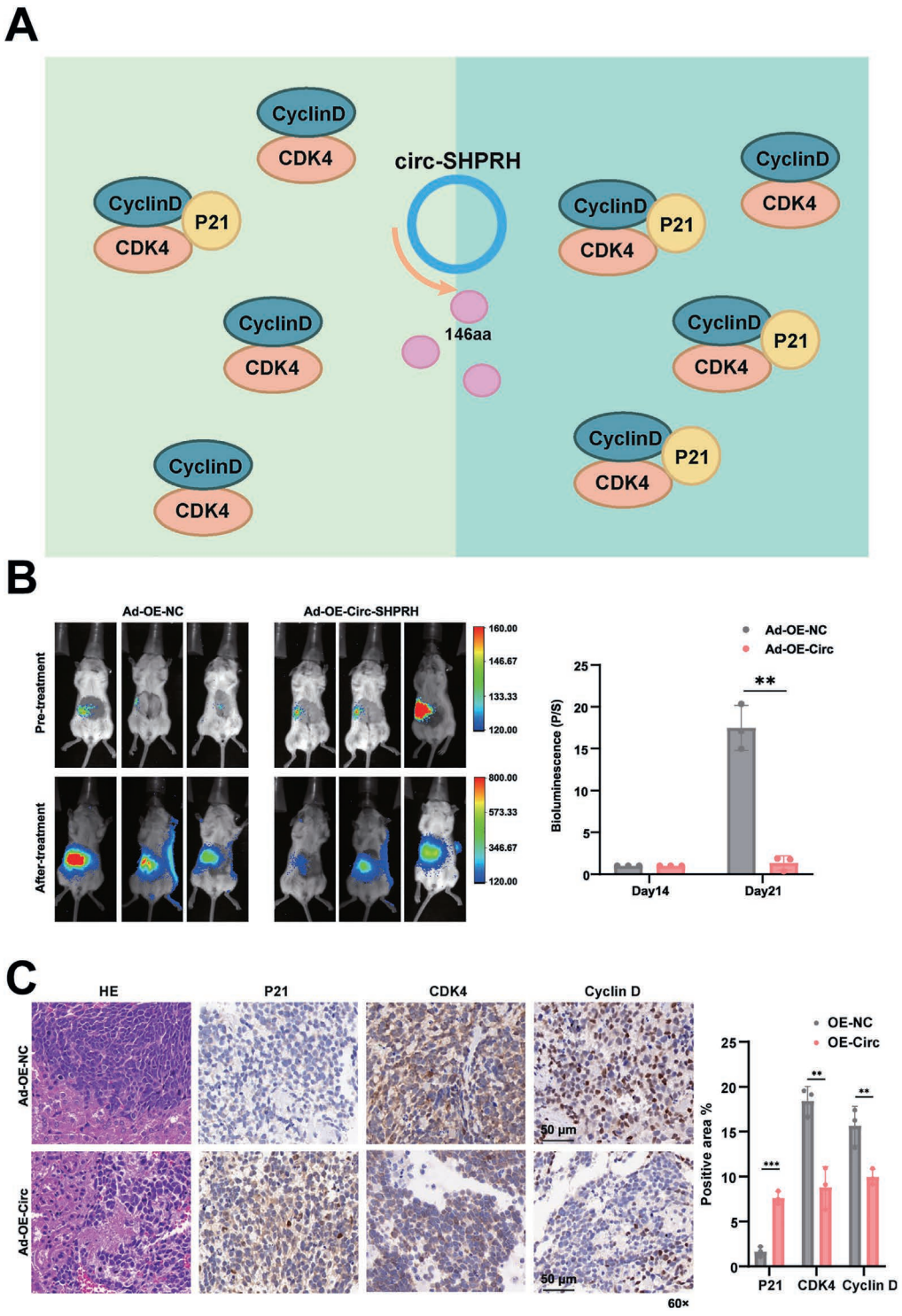

Figure 5 The protein-coding potential of circRNAs. (A) circ-SHPRH-encoded therapeutic SHPRH-146aa dynamically upregulates P21 to inhibit CDK in neuroblastoma. (B, C) Tumor inhibitory effects of administered circ-SHPRH. Adapted with authorization from Ref. 117 © 2024 Elsevier B.V. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

fold the translation efficiency compared to traditional circRNA[120]. The intrinsic stability helps to prolong the protein translation, lengthen the antigen presentation, and prolong the protein expression cycles, significantly improving the efficacy of multi-field treatment technologies. Continuous antigen synthesis supports sustained effector responses and enhances the intensity of host immune engagement. The cap-independent translation remains unaffected by IFN, avoiding inhibition that typically follows Th1-driven immune response[121].

circRNA provides unique advantages for the stable delivery of tumor antigens owing to its nuclease resistance and long-term expression conferred by its closed-loop structure. Research has

confirmed that the circRNA-based antigen-encoding system can significantly enhance adaptive immunity (Fig. 6). By synthesizing an engineered circRNA encoding chicken ovalbumen (OVA) *in vitro*, it successfully expanded OVA-specific $CD8^+$ T cells and achieved a notable tumor suppression in a melanoma mouse model, highlighting the strong immunogenic potential of circRNA vaccines. Wang et al. [122] innovatively constructed an anti-HER2 CAR circRNA platform that simultaneously encoded both CAR elements and HER2 antigen epitopes through a single-stranded design. In a co-transfection setting involving T cells and macrophages, this strategy achieved CAR/HER2 coordinated expression by immune cells, promoted the cross-presentation of tumor-associated antigens, and eliminated $HER2^+$ tumor cells, indicating the feasibility of circRNA-mediated immune activation as an antitumor strategy.

A core challenge in tumor immunotherapy is the inherently low mutational burden of many solid tumors, resulting in extremely limited high-specific neoantigen peptides and restricting the efficacy based on antigen recognition[123]. Tumor-derived circRNA-encoded peptides have received attention due to their heightened immunogenicity and potential to generate novel epitopes. Researchers are actively developing programmable novel delivery systems for specific antigen peptides. Through sustained antigen production, circRNA enables personalized treatment adaptation, leverages its continuous expression to dynamically generate new antigens, promotes antigen presentation and immune activation, and effectively mitigates immune escape and tolerance. Multiple studies have developed circRNA neoantigen vaccines that obtain more durable and robust immune responses compared to linear RNA. Wang et al.[124] designed a circRNA-Ptpn2_I383T vaccine targeting liver cancer, which maintained antigen expression *in vivo* for more than 11 days, and efficiently promoted dendritic cells maturation as well as infiltration of specific T cells, achieving a higher inhibitory effect on carcinoma *in situ*. Meanwhile, Song's team[69] focused on concealed antigens in breast cancer and used LNP to deliver hidden peptides encoded by circFam53b, which could bind to HLA-I and specifically activate $CD4^+$ and $CD8^+$ T cells, and enhanced immune-cell infiltration and subsequent tumor suppression. These studies jointly verify that circRNA antigen vaccines can break through the classical barrier of immune tolerance by extending the antigen exposure and enhancing cross-presentation efficiency. The intensity and persistence of T cell responses induced by these vaccines are significantly superior to those of traditional linear RNA platforms, positioning new antigen circRNA vaccines as a new immunotherapy strategy.

CircRNA-based tumor vaccines have progressed to the clinical trial stage. Zhang et al.[125] developed a multivalent circRNA vaccine, Apt-circRNA-KR2, specifically targeting *KRAS* G12D. This vaccine demonstrated highly efficient lymph node targeting and significant anti-cancer effects through direct injection. Subsequently, the team conducted a dose-escalation safety assessment involving nine healthy volunteers, followed by a repeat-dose trial with three additional healthy subjects. Notably, only one subject experienced transient flu-like symptoms, indicating good safety profiles for the vaccine. Concurrently, scRNA-seq and transcriptomic analyses of peripheral blood mononuclear cells from the three subjects revealed an increase in immune cell populations

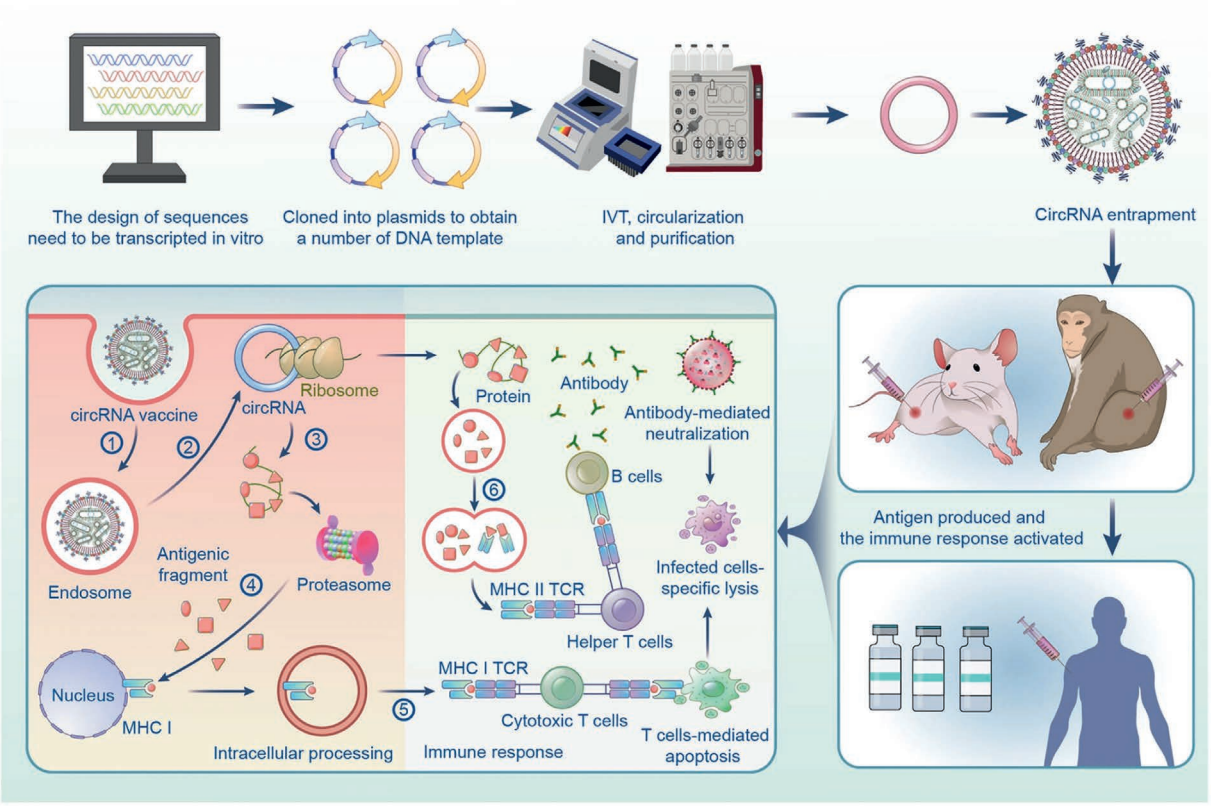

Figure 6 Development process of circRNA vaccines and mechanisms of immune response. Adapted with permission from Ref. 96 © 2023, The Author(s).

post-vaccination, alongside hundreds of differentially expressed genes in myeloid cells, T cells, and B cells, along with enrichment of inflammatory signaling pathways. These findings provide initial evidence that the vaccine successfully induced specific immunity against the targeted antigen[125]. These preliminary results mark a significant transition to human trials and demonstrate the safety of the circRNA vaccine. Future systematic clinical trials will be necessary to further evaluate its efficacy.

Cytokines are recognized as pivotal factors in orchestrating interactions between tumors and immune cells. High-dose cytokine therapy can activate immune cells or directly induce apoptosis in tumor cells, and combination strategies with targeted therapy and immunomodulators have achieved better therapeutic outcomes. The coding capability of circRNA provides a versatile platform to achieve multi-dimensional regulation of antigen presentation, T cell activation, and immune microenvironment remodeling by continuously expressing immunomodulatory factors. Multiple studies have achieved higher immune activation based on the elevated production of cytokines by circRNA. Yang et al.[126] constructed cmRNA based on an IRES from echovirus 29, directly injected cmRNA encoding IL-12, IL-15, GM-CSF, and IFNA2 into mixed tumors. PD1 antibody was also administered for synergistic treatment. The results demonstrated that both single-agent and combination therapies could significantly inhibit tumor growth. The synergistic effect of cmRNA and PD1 antibody further enhanced the remodeling of the immune microenvironment and achieved the highest level of immune infiltration. He et al. [127] has developed a cyclized IL-23 molecule with an intrinsic immune-cascade amplification effect. Encapsulated with LNP and co-transfected with the stimulator of interferon genes agonist MSA-2-Pt achieved tumor suppression following a single injection. Active targeting and intratumoral injection techniques such as LNP facilitate high local cytokine expression while mitigating the risk of inflammatory storms that can arise from excessive cytokine accumulation in the bloodstream, thereby ensuring safety while enabling high levels of inflammatory factor expression.

3.4. Application of exogenous circRNA in gene editing

Gene editing technology based on the CRISPR system provides a transformative approach for tumor treatment. It achieves precise knockout of specific genes by delivering Cas-protein-encoding mRNA together with sgRNA, allowing precise knockout of tumor-relevant genes while avoiding the systemic toxicity and repeated dosing required by conventional treatments. The CRISPR platforms are also used in genome-wide screening to identify new therapeutic targets. However, the clinical transformation is limited by the nuclease sensitivity of sgRNA and the size-dependent off-target effect associated with Cas9[128]. To tackle the issue of sgRNA stability, circRNA technology has shown unique value: transforming linear sgRNA into covalently closed circRNA can significantly increase its half-life and sustain the genome-editing activity. At the same time, the discovery of Cas12a has higher targeting accuracy in tumor genome editing due to its preference for A/T-rich sequences and smaller molecular weight (~1277 aa *vs.* Cas9's ~1368 aa). However, its intrinsic pre-crRNA cleavage activity renders conventional linear prime editing guide RNA unsuitable[129]. Gao's team[129,130] recently developed an innovative circRNA-mediated multi-target guide editor utilizing Cas12a. By connecting the CRISPR RNA from four different systems (nuclease-dependent, nickase-dependent, split nickase-dependent, split nuclease-dependent) in series onto a single circRNA backbone, it can efficiently edit 2 to 4 gene sites with minimal detectable off-target effects. This circRNA-based Cas12a guide system has expanded the utility of Cas12a in both fundamental tumor research and gene therapy (Fig. 7A).

Adenosine deaminases that act on RNA (ADAR)-based RNA editing is a reversible approach that circumvents the introduction of permanent mutations, thus avoiding genetic alterations associated with off-target effects[131]. The mechanism relies on an oligonucleotide with an antisense targeting sequence, which recruits endogenous ADAR to form an editing complex, which converts A-to-inosine(I) conversion in target transcripts. It is subsequently interpreted as guanosine (G), enabling precise A-to-G editing[132]. Traditional sgRNA has shown inefficiency in recruiting native ADARs, necessitating the exogenous ADAR supplementation. Katrekar et al.[133] designed a circular ADAR recruitment guide RNA that enhances ADAR binding affinity and higher stability, and longer activity compared to linear sgRNA. This circular structure enables guide RNA to form a closed-loop structure within the target transcripts, enabling highly efficient RNA editing with no need for exogenous ADAR. Editing can be performed on specific RNA sequences to inhibit tumor cell proliferation or promote anti-tumor immunity. At present, the role of ADAR in tumors remains controversial: although early studies categorized ADAR as an oncogene due to its involvement in cancer-promoting pathways depends on the editing effect of ADAR1, recent findings have revealed that ADAR's editing activity exerts a bidirectional regulatory effect on various tumors. For example, in breast cancer, ADAR re-editing of *GABRA3* transcripts inhibits the proliferation and migration of tumor cells. In addition, the high abundance of ADAR within the tumor microenvironment provides an opportunity for RNA supplementation. Wang et al.[134] delivered exogenous circ-arRNA into triple-negative breast cancer cells to recruit high-abundance ADAR to edit the nonsense-mutated *TP53-W53X* transcript. This intervention effectively replaced the stop codon, successfully restored protein expression, and sensitized tumor cells to albumin-bound paclitaxel (Fig. 7B). These findings underscore the importance of precise design and selection of specific signaling pathways and target genes when applying ADAR-dependent RNA editing technology to fully exploit their therapeutic potential[135,136].

4. circRNA delivery vectors

4.1. LNPs for exogenous circRNA delivery

LNPs have emerged as one of the most advanced and widely employed vectors for nucleic acid delivery, with their significance notably highlighted during the COVID-19 pandemic[137]. LNP-based mRNA vaccines for COVID-19 have not only enabled a large-scale vaccination effort but also driven significant advancements in the LNP system with RNA therapeutics[17]. Structurally, LNPs are mainly composed of four components: ionizable lipids, phospholipids, cholesterol, and polyethylene glycol lipids (PEG-lipids), which are co-assembled in a certain ratio. The alkylated quaternary ammonium groups of ionizable lipids remain neutral at physiological pH (~7.4), minimizing nonspecific interactions with RNA. In the acidic environment of the endosomal body (pH 5−6), these lipids rapidly protonate after being positively charged, and interact with the negatively charged membrane of the endosomal body, facilitating membrane fusion and releasing

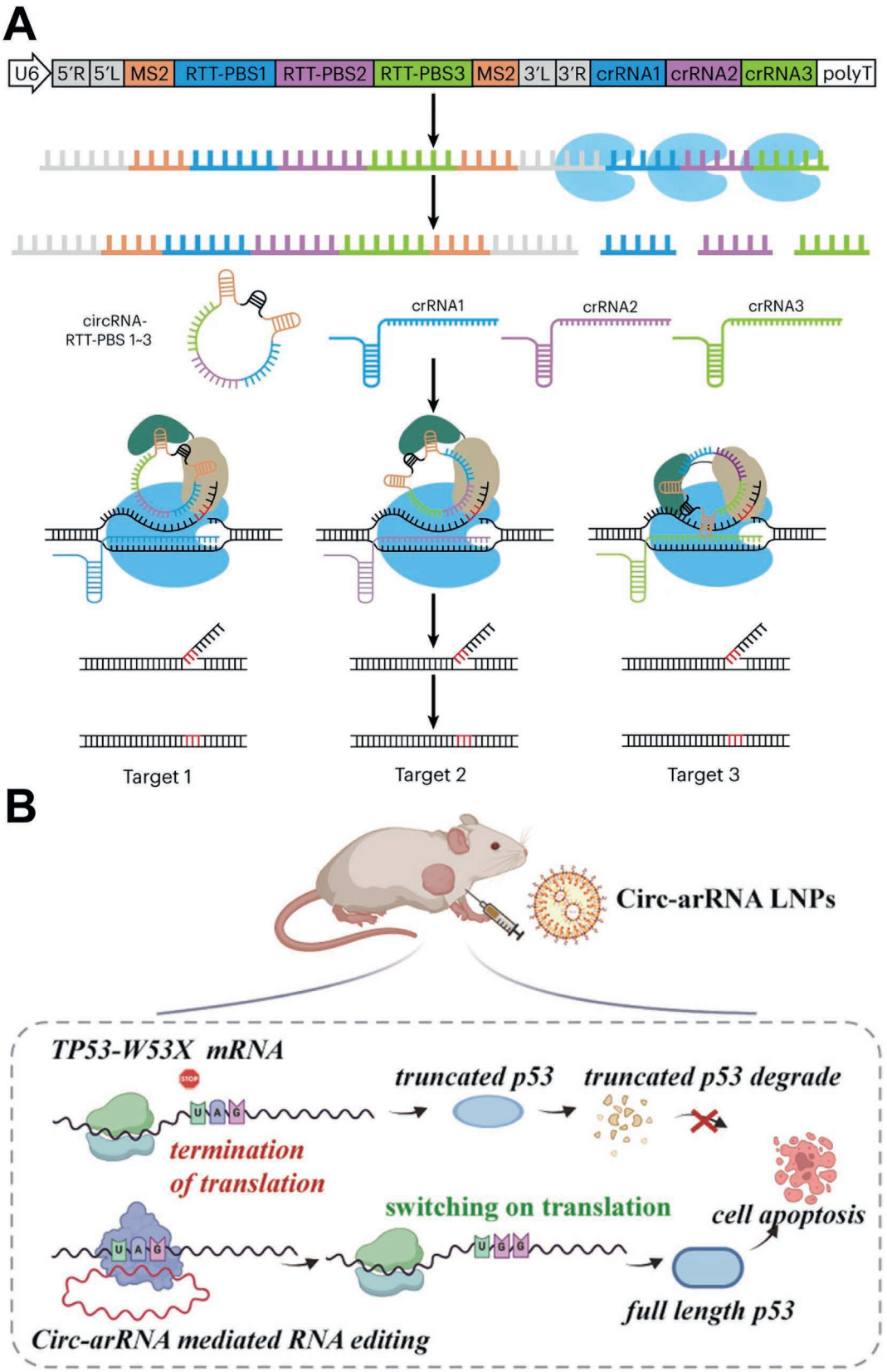

Figure 7 Applications of circRNA in precise gene and RNA editing. (A) Construction of circRNAs incorporating multiple crRNAs, combined with CRISPR-12a for the simultaneous editing of multiple genes. Adapted with permission from Ref. 130 © 2024, The Author(s), under exclusive license to Springer Nature America, Inc. (B) Development of a circular ADAR-recruiting RNA (Circ-arRNA) designed for *in vivo* RNA editing-mediated correction of TP53 nonsense mutations. Adapted with permission from Ref. 134 © 2025, American Chemical Society.

RNA. Phospholipids and cholesterol regulate the stability and fluidity of the particles, while PEG-lipid is used to optimize the circulation half-life and limit protein adsorption[138,139]. LNPs are typically self-assembled by rapidly mixing a lipid-containing organic phase with an RNA aqueous phase. Techniques such as microfluidic mixing, T-junction mixing, and rapid injection allow precise control over particle size (usually 50−150 nm) and polydispersity index by controlling lipid concentration, stirring rate, and flow rate ratios (Fig. 8A)[140−142]. In particular, microfluidic preparation enables large-scale production of uniform size with the mRNA encapsulation rate exceeding 95%[143]. With excellent biocompatibility, high loading capacity, and suitability for industrial production, LNPs have found extensive use for the *in vivo* delivery of mRNA, siRNA, and recently circRNA.

Compared with traditional linear RNA, the circular structure provides superior stability and prolongs more persistent protein expression, and reduces susceptibility to immune recognition. However, LNP delivery also brings new challenges due to circRNA's long, rigid cyclic chains. This necessitates optimization of the lipid formula, fine-tuning nucleic acid-to-excipient ratios, and improving microfluidic parameters, such as enhancing shear force to ensure sufficient encapsulation rate and narrow particle size distribution. Once internalized by cells, the release of circRNA in an acidic environment is often delayed, requiring the use of more sensitive ionizable lipids to promote membrane destabilization. At present, the research on circRNA carriers has also expanded beyond basic preparation toward improving delivery efficiency, enhancing targeting, and regulating immunogenicity.

An increasing number of novel components are incorporated into LNP formulations, with promising results demonstrated in multiple tumor models. For example, Xu et al.[144] introduced a novel ionizable lipid H1L1A1B3 into LNPs. Although these LNPs preserved comparable physicochemical profiles to mRNA-LNPs, a single *in vivo* injection significantly enhanced circRNA delivery to lung cancer cells, achieving a fourfold increase compared to the industry standard LNP, and enhancing $CD45^+$ and $CD8^+$T cell infiltration. H1L1A1B3 also activated immune pathways through the NFKB/IRF pathway, further expanding the application potential of LNP in RNA immunotherapy (Fig. 8B-D). PEG-lipid, a widely used component to prolong blood circulation, can induce anti-PEG antibodies, thereby accelerating LNP clearance and diminishing delivery efficiency. To solve this bottleneck, Kang et al. [145] replaced PEG-lipid with polysarcosine lipid (pSar-lipid) in the formulation of LNPs with SM102 or ALC-0315 as an ionizable lipid. This modification achieved delivery efficiency and immunogenicity comparable to traditional PEG-LNP, while significantly reducing the risk of allergic reactions. LNPs are usually prepared in lyophilized form for long-term transport and storage. However, the mechanical stress during lyophilization and reconstitution will cause irreversible damage to the morphology and structure of LNP, interfering with the delivery efficiency[146]. Wan et al.[147] demonstrated that mannose-modified LNP retained its physical properties post-lyophilization. Mannose modification also mediated active uptake by dendritic cells, enhancing the lymph node targeting. This modification prolonged the antigen expression and a more sustained specific immune response. In summary, innovations in LNP-based delivery are advancing through the sequential stages of ingredient design, function optimization and targeted delivery, offering an expandable and customizable high-performance carrier platform for circRNA therapy.

4.2. *Exosomes for exogenous circRNA delivery*

Exosomes are nanoscale extracellular vesicles secreted by a variety of cell types and detectable in most tissues and body fluids. Exosomes feature a characteristic lipid bilayer structure with diameters ranging from 40 to 160 nm. Due to their derivation from autologous or homologous cells, exosomes naturally exhibit excellent biocompatibility and low immunogenicity[149]. Exosomes can traverse multiple biological barriers such as the blood−brain barrier and basement membrane, facilitating efficient intracellular transport and signal dissemination[150]. More importantly, exosomes are naturally rich in endogenous circRNAs, with over 1000 species having been identified in human serum, largely due to the structural protection afforded by the vesicle. In contrast to mRNA delivery, the closed-loop structure of circRNA requires a carrier with higher stability and targeting capabilities, which makes exosomes an ideal choice for delivering circRNA due to their natural adaptability[151,152]. The exosomal membranes contain abundant integrins and tetraspanins (such as CD63, CD81), which facilitate adhesion and uptake by recipient cells. Tumor cells naturally have a higher internalization efficiency of exosomes, a mechanism exploited during the transmission of drug resistance. This characteristic also suggests that tumor-derived exosomes possess a high degree of adaptability to tumor environments, allowing them to be modified for the delivery of tumor suppressive circRNA[153]. CircRNA can be enriched in donor cells *via* plasmid transfection, or synthetic circRNA can be loaded into extracted exosomes by electroporation, sonication, or related methods. Downstream purification typically uses ultrafiltration, extrusion, microfluidics, or membrane fusion. However, unlike natural tumor-encapsulation exosomes, exogenous circRNA delivery often faces the dilemma related to loading efficiency and large-scale manufacturing. The rigid and spatially bulky cyclic structure of circRNA limits payload capacity, while reliance on cell-based exosome production compromises yields and purity. Artificial encapsulation methods also risk poor vesicle stability and issues with membrane protein fusion (Fig. 9A). As a result, efforts to enhance loading efficiency, purification, and internalization are under intensive investigation. For example, Pan et al.[154] demonstrated that hnRNPA2B1, a key regulator of RNA encapsulation, can also bind to circRNA and promote encapsulation into exosomes, which aids in packaging circRNAs that lack natural sorting signals. Similarly, Alvarez et al.[155] engineered a targeted exosome by fusing specific peptides to the N-terminus of Lamp2b, a membrane protein abundantly expressed in exosomes, enabling the targeted delivery of these vesicles. The ligand can also be directly conjugated onto exosomal membranes. For example, poly-D-lysine-graft-triphenylphosphine, as a mitochondrial targeting signal, can be electroporated into exosomes *in vitro*, enabling effective mitochondrial targeting in macrophages[156-158]. Currently, numerous studies are exploring the use of recombinant circRNA derived from exosomes for tumor treatment. Recombinant exosomes engineered to express the tumor suppressor circRNA-CREIT have been constructed and administered *via* the tail vein injection. CircRNA-CREIT sensitizes tumor cells to chemotherapy by interfering with EIF2S1 phosphorylation and stress granule formation (Fig. 9B)[57].

4.3. *Viral vectors for exogenous circRNA delivery*

VLPs are spherical nanocarriers that self-assemble from single or multiple viral structural proteins, including nucleocapsid and

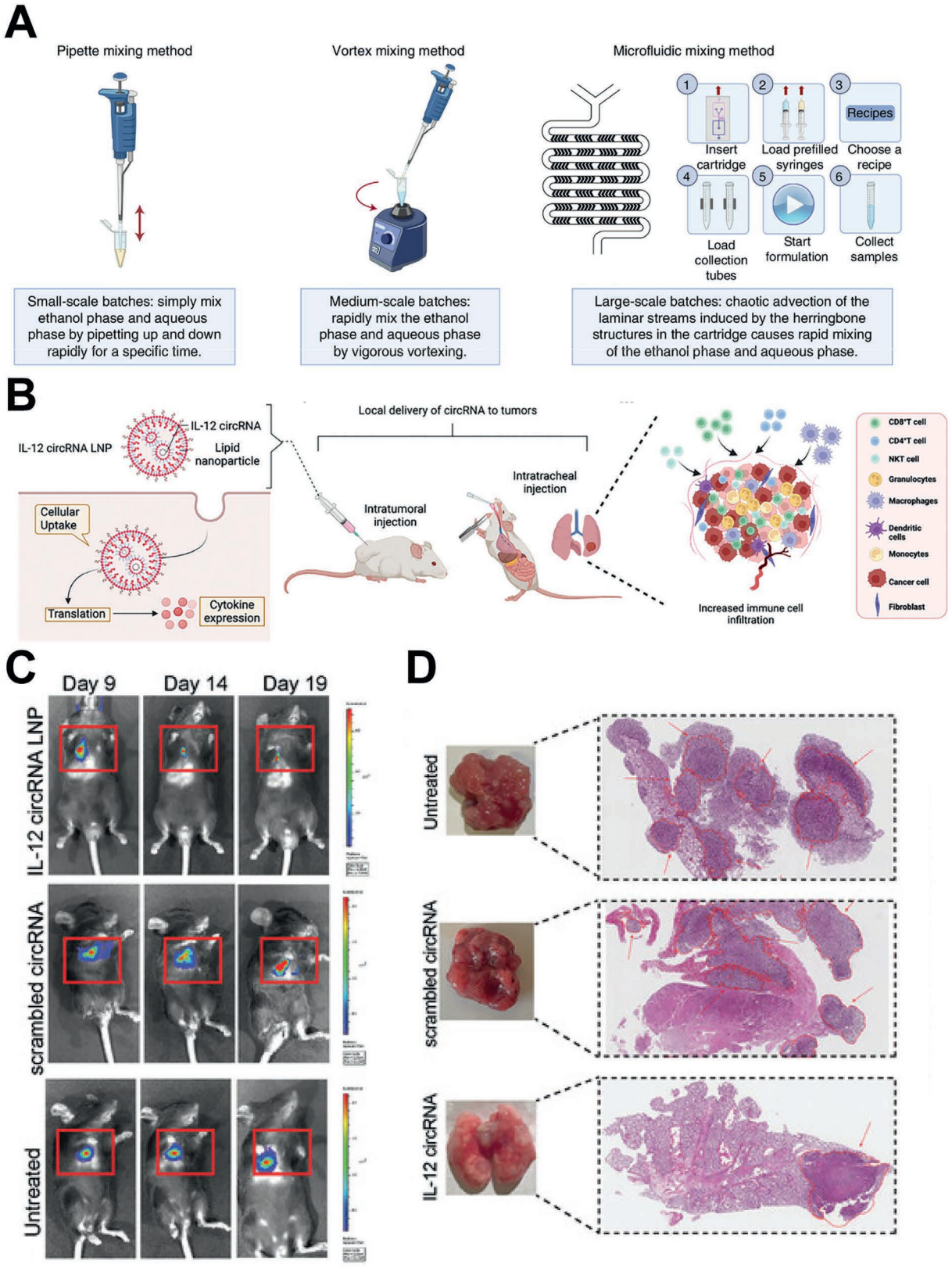

Figure 8 Lipid nanoparticle (LNP) platforms for circRNA delivery. (A) Preparation of LNPs through pipette mixing and vortex mixer microfluidic techniques. Adapted with permission from Ref. 148 © 2022, Springer Nature Limited. (B) Delivery and translation of IL-12 circRNA LNPs in tumor cells. (C, D) Intratracheal administration of IL-12 circRNA LNPs significantly reduces lung tumors in mice. Adapted with permission from Ref. 144 © 2024 Wiley-VCH GmbH.

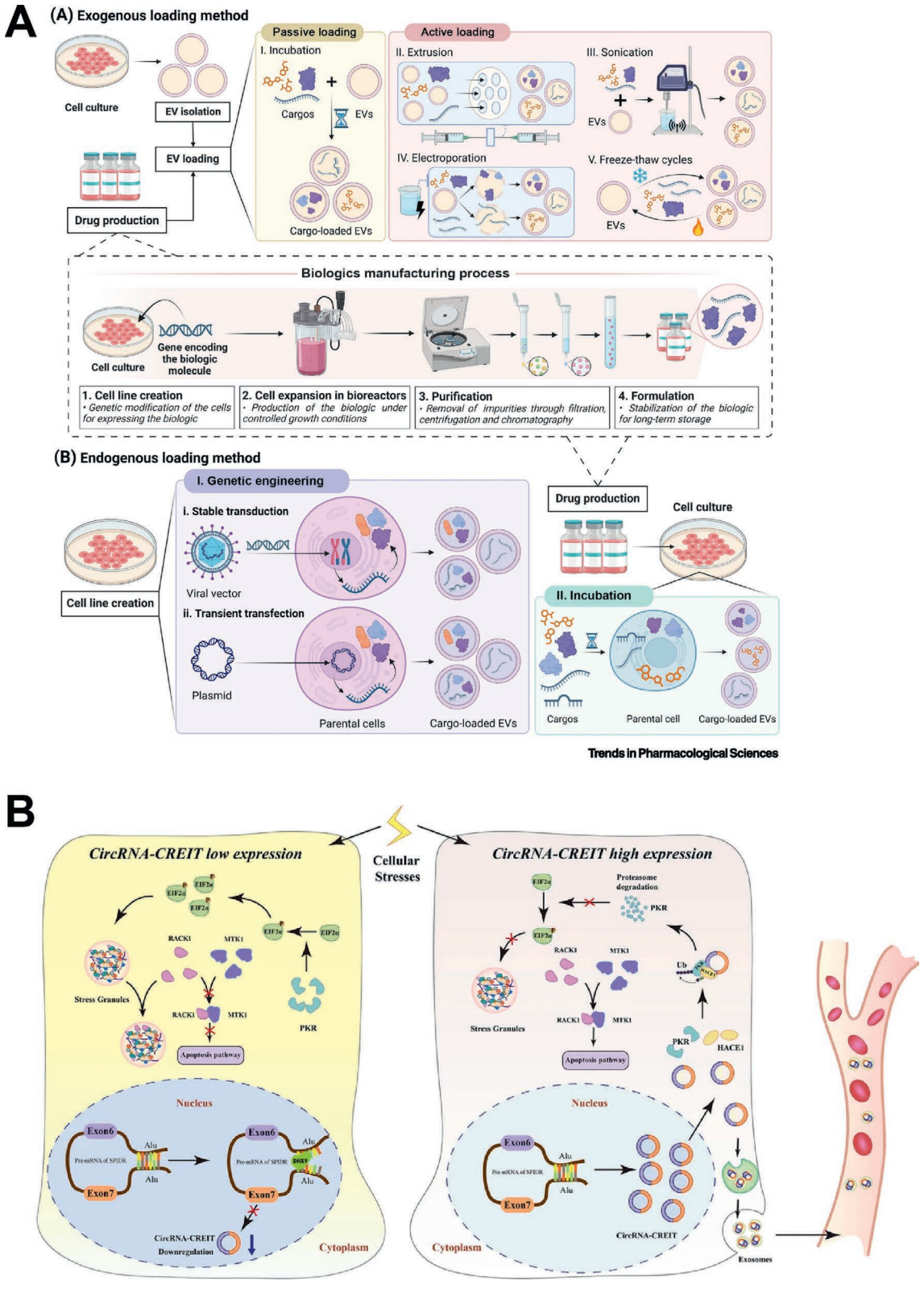

Figure 9 Exosome-mediated delivery and loading strategies for circRNAs. (A) Exosome loading methods for drug delivery. Adapted with permission from Ref. 159 © 2024 Elsevier Ltd. All rights reserved. (B) CircRNA-CREIT delivered *via* exosomes inhibits the formation of stress granules, thereby suppressing chemoresistance in triple-negative breast cancer. Adapted with permission from Ref. 57 © 2022, The Author(s).

envelope proteins, under both *in vitro* and *in vivo* conditions. They closely resemble natural viruses in morphology but lack any replication-related viral nucleic acids[160]. The hollow internal structure enables efficient encapsulation of cargoes. *In vivo* synthesis of VLPs typically involves introducing viral structural genes in systems such as *E. coli*, yeast, or plant cells, followed by protein self-assembly, centrifugation, and purification. During assembly, lipid layers derived from the host cell membrane may also be incorporated when VLPs bud through the membrane[161]. *In vitro* assembly utilizes purified capsid proteins in a cell-free environment, where spontaneous polymerization occurs by modulating factors such as ionic strength, pH, or adjuvants[162]. Structural proteins can originate from a single virus type or be engineered as chimeric forms composed of multiple viruses. Since VLPs carry no viral genes, they exhibit excellent safety profiles. Moreover, these particles consist predominantly of highly immunogenic viral proteins, which can serve as natural adjuvants to elicit robust humoral and cellular immune responses *via* major histocompatibility complex pathways[163]. Proteins such as Vesicular stomatitis virus glycoprotein and Env can be incorporated into VLPs by pseudo-typing surface proteins to enhance carrier-cell interactions. Targeting ligands can be added to enable selective uptake. Lipid components may further improve the stability[164]. Compared with other delivery systems, VLPs have the unique capability to deliver RNA directly into the cytoplasm, bypassing endosomes and reducing endosomal losses, and improving expression efficiency. These advantages make VLP a potential delivery vector for circRNA. Efficient circRNA delivery relies on appropriate protein-sequence design to facilitate correct self-assembly and maintain stability. This is particularly critical for incorporating chimeric constructs, where it is essential to preserve the membrane positioning and folding of the original structural proteins to ensure proper VLP encapsulation and cell−cell interactions. Whether VLPs are generated by budding or cell lysis, they often carry various intracellular components, including cellular debris, cell-derived proteins, nucleic acids, lipids, and impurities from the culture medium. Such contamination can compromise purity and therapeutic efficacy. Therefore, purification constitutes a crucial step in ensuring stability and immunogenicity. This process typically employs methods such as ultrafiltration and affinity chromatography to eliminate residual impurities. A major bottleneck in circRNA delivery is its relatively large, rigid circular structure and the absence of sorting sequence signals. To address this issue, Unti et al.[116] applied the "Tornado" system by introducing autocatalytically cleavable Twister ribozyme sequences flanking the RNA sequence. This modification allows the transcription products to generate 5′-hydroxyl and 3′-2′,3′-cyclic phosphate ends, which subsequently facilitates efficient cyclization by the RtcB enzyme. CircRNA produced by this system, such as the CMV-CVB3 circRNA, exhibited high intracellular expression and was effectively packaged into VLPs, significantly prolonging protein expression (Fig. 10A-C). These studies provide novel insights into the efficient and precise delivery of circRNA, positioning VLP as a promising platform for future therapies.

Viral vectors, especially AAV, are naturally utilized for DNA transfection. When delivering circRNA, it is essential that they facilitate *in vivo* transcription and circularization *via* DNA intermediates. Recombinant AAV is widely used due to its non-pathogenicity, low immunogenicity, and no genome integration[165]. During the construction process, the genetic encoding of the circRNA precursor, which contains a base intron to promote reverse splicing, is inserted between the inverted terminal repeats of the viral genome. Following cellular transduction, host RNA polymerase II drives transcription of the precursor transcript, which subsequently generates closed-loop RNA. Meganck et al.[166] developed a "split-GFP" loop system where the fragmented GFP coding sequence was placed between two introns. This configuration allowed for restoration of the full-length GFP sequence and achieved a continuous IRES-dependent mechanism. Stable and persistent GFP expression has been observed in various tissues such as the mouse heart, eyes, and other parts. AAV-circRNA also shows therapeutic potential in oncology. Ding et al.[113] successfully encapsulated tumor suppressive circRERE in AAV and significantly inhibited tumor growth in CRC mouse model (Fig. 10D-E). However, the AAV-mediated system requires precise control over reverse splicing sites and IRES configurations; otherwise, it is prone to produce undesirable linear splicing events, diminishing both the yield and functionality. Unlike transient RNA carriers, the transposon system furthermore offers a stable integration and expression of circRNA. The researchers transiently expressed piggyBac or Sleeping Beauty transposase to integrate circRNA constructs containing circularization elements into the cell genome, successfully inducing high-level and persistent circRNA expression in *in vitro* cell lines and mouse tumor models without the need for persistent viral infection[167].

4.4. Other vectors for exogenous circRNA delivery

In addition to the delivery carriers mentioned above (Table 2), several delivery platforms initially developed for linear RNA have now been adapted for circRNA. Charge-altering releasable transporters (CART), a polymer composed of carbonate-b-α-amino ester, are prepared by oligomerization and deprotection of lipid-based and cationic monomers. CART has almost no inherent immunogenicity and undergoes charge rearrangement to form a neutral diketopiperazine molecule under physiological pH, which eliminates the electrostatic force with RNA. This enables efficient RNA release while avoiding the cytotoxicity associated with cationic materials[168]. Distinct from liver-enrichment systems such as LNP, intravenously administered CART demonstrates highly selective targeting to specific organ tissues, primarily the spleen and lung, without requiring targeting ligands or chemical modifications. Moreover, when administered *via* intramuscular injection, CART restricts expression to the injection site without leakage. The synthesis is relatively straightforward, and it can be stably stored in DMSO at -20 °C for over one year, making it convenient for clinical application[169]. The GSer-CART platform developed by Li et al.[170] achieved efficient delivery of circRNA encoding ovalbumin, elicited a potent $CD8^+$ T cell response, and significantly suppressed tumor growth in models, highlighting the promising potential of CART in circRNA delivery, especially in the field of tumor immunotherapy.

Inorganic nanomaterials are increasingly recognized as appealing candidates for nucleic acid delivery due to their precise size tunability, ease of surface modification, and exceptional stability. Among these, gold nanoparticles (AuNPs) have garnered significant attention in RNA delivery due to their favorable biocompatibility, controllable synthesis, and versatile surface chemistry. RNA can be readily loaded onto the surface of AuNPs through thiol bonds or other non-covalent forces, while the preferential adsorption of poly-A sequences further facilitates robust RNA binding[171]. Particles with varying diameters can be

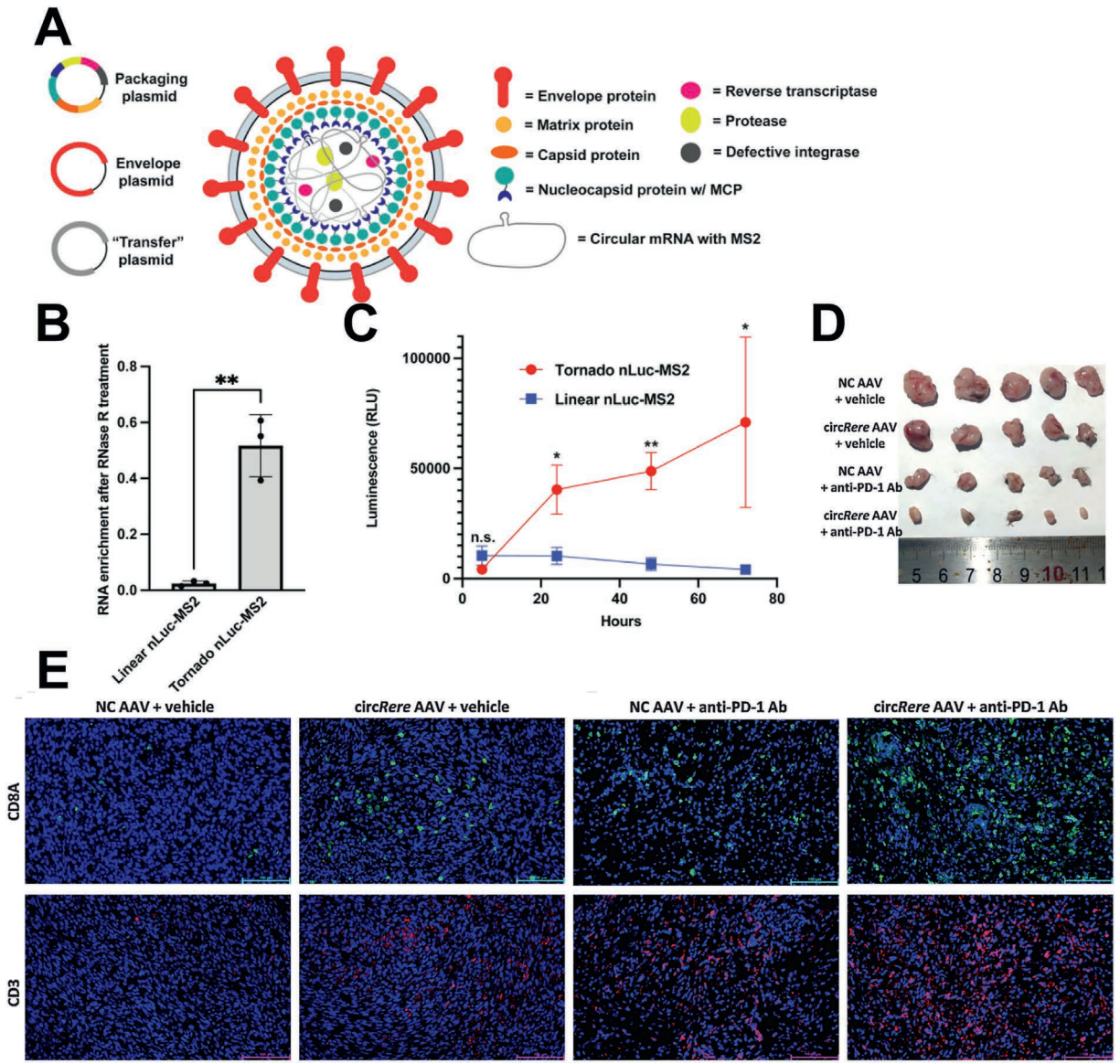

Figure 10 Viral and virus-like particle (VLP) vectors for circRNA delivery. (A) Schematic representation of circRNA-VLP loaded with the Tornado translation system. (B, C) CircRNA is identified within VLPs packaged by the Tornado translation system, demonstrating an extended duration of protein expression. Adapted with permission from Ref. 116 © 2023 Elsevier Ltd. (D, E) The group treated with AAV-encapsulated circRERE in combination with anti PD1 antibody exhibits the highest level of tumor suppression, accompanied by a significant increase in $CD8^+$ and $CD3^+$ T cell infiltration. Adapted with permission from Ref. 113 © 2023, American Association for Cancer Research.

accurately produced by simply adjusting the molar ratio of gold salt to reducing agent. *In vivo* studies indicate AuNPs can accumulate in tumor tissues *via* the enhanced permeability and retention effect. Their high surface area allows for the functionalizing of various ligands to enhance member interaction and cellular rates. Furthermore, the inherent optical absorption properties enable integration of diagnostic and therapeutic modalities such as near-infrared photothermal therapy and photoacoustic imaging. At present, loading circRNA expression plasmids onto AuNP surfaces has emerged as a common and efficient strategy for delivery[54]. However, the potential toxicity of AuNPs is closely related to their particle size. While increasing surface charge enhances cellular uptake, it may also result in nonspecific protein adsorption, compromising targeting specificity and biodistribution. These considerations highlight the need for careful optimization and balancing in subsequent designs.

5. circRNA therapy and artificial intelligence

The rapid advancement of AI and computational biology is transforming traditional biomedical research, especially in the design and optimization of RNA delivery systems. Across multiple dimensions such as active site screening, structure prediction, component optimization, pharmacokinetic simulation, and delivery platform engineering, AI is expected to significantly improve the *in vivo* stability and expression efficacy of the circRNA systems, while also achieving personalized precision treatment[175,176].

Table 2 Comparison of different delivery systems.

Platform	Loading strategy	Targeting modifications	Immune activation	Scalability	Stability	Limitations	Representative uses	Ref.
LNP	Direct nucleic acid encapsulation	Ligand-decorated lipids	Low−moderate innate activation	High	Cold chain; lyophilizable	Liver accumulation; acute toxicity concerns	Approved mRNA vaccines (*e.g.*, Comirnaty; mRESVIA)	137,144,147
Exosome	Endogenous secretion or *ex vivo* loading	Surface ligand display	Low; variable (xenogeneic)	Limited; purification challenging	Cold storage; lyophilizable	Low yield; limited industrial readiness	Preclinical	57,150,154,155
VLP	Self-assembly of viral structural proteins	Capsid peptide insertion	Intrinsically immunogenic	High	Stable; lyophilizable	Neutralizing antibodies; high innate activation	HPV vaccines (*e.g.*, Cervarix)	161-163
AAV	Packaging of therapeutic genome	Capsid engineering variants	Immunogenic capsid and anti-AAV antibodies	High	Cold storage	Pre-existing antibodies; integration	Gene therapies (*e.g.*, Luxturna)	165,166

AAV, adeno-associated virus; LNP, lipid nanoparticle; VLP, virus-like particle.

AI significantly expedites the identification of disease-associated circRNA and other regulatory molecules. By leveraging deep learning techniques and self-supervised feature extraction, AI can identify circRNA molecules closely linked to disease phenotypes from multi-omics datasets. Algorithms such as graph neural networks and convolutional neural networks are widely employed to analyze single-cell spatial transcriptomic data to construct heterogeneous network models for circRNA-miRNA and circRNA-disease interaction networks, unveiling circRNAs that are intricately involved in pathological progression within the complex microenvironment of tumors[177]. The predictive capabilities can greatly reduce analytical burdens while improving the accuracy in relevant predictions (Fig. 11A)[178]. Systematic screening of circRNAs enables the establishment of therapeutic strategies, such as silencing for cancer-promoting circRNAs or delivering cancer-suppressing circRNAs, and facilitates the development of prediction and prognosis models based on variations in circRNA abundance. AI also plays a pivotal role in the rapid identification of immunogenic targets. Genomic and proteomic analysis of tumor samples enables the isolation of key mutant proteins. The discovery of immunodominant epitopes that can reliably elicit specific immune responses is crucial for tumor vaccines. Classical bioinformatics tools such as SYFPEITHI and NetMHC, powered by neural network algorithms, can accurately predict antigen affinity and structural characteristics across various immune cells, including macrophages, dendritic cells, B cells, and T cells. These tools support the rational design of antigen-encoding circRNAs that maximize immune activation while minimizing cross-reactivity and off-target effects (Fig. 11B)[179,180]. Furthermore, integrating the spatial expression map with the immune phenotype enhances the tissue-specific recognition capabilities and lays a foundation for the precise encoding of circRNA.

During the optimization phase, codon optimization tools such as JCAT and Gene Optimizer refine coding regions according to host-specific codon preference, CG content, and secondary structure, which aids in generating more stable transcripts while improving translation efficiency. Structural prediction software such as RNAfold contributes to the design of highly stable active circRNA as an essential factor in maintaining structural integrity and ensuring encapsulation and delivery within nanocarriers[181,182]. Thoughtful RNA structure design expands the active sites available for interaction with miRNA and RBPs, thereby maximizing target adsorption and enhancing silencing efficiency.

AI is increasingly utilized in the screening of nanocarrier components and the analysis of drug dynamics. The rigid structure of circRNA limits the encapsulation of LNP and EVs. For LNP, various design tools such as NANO design, POLYVIEW-3D, and COMSOL serve as effective screening platforms to simulate key formulation parameters, including lipid composition and molar ratios, particle size, polydispersity index, and surface charge. These simulations facilitate the identification of the optimal formulation for *in vivo* delivery. These tools allow for the visualization of nanoparticles' 3D morphology and further simulation of interaction with biological structures such as plasma membranes and endosomal compartments. Such modeling provides mechanistic insights involved in RNA uptake and release, guiding the optimization for LNP production[175,183]. AI also supports experimental design optimization. By integrating multi-omics data to create a feedback optimization model. AI enables the dynamic updates of circRNA expression levels, mutant antigen peptides expression, immune activation states, and formulation variables, including lipid libraries. This approach accelerates the optimization of both circRNA sequences and nanocarrier architectures, ultimately achieving candidate screening and reducing experimental burden. AI can also develop predictive models using polymer databases, incorporating factors such as component ratio, physicochemical properties, antigen loading capacity, and excipients to investigate drug-release kinetics, enabling rapid preselection of promising delivery materials[184]. Overall, AI and biocomputing are revolutionizing the delivery of exogenous circRNA with remarkable speed and accuracy, establishing a robust foundation for realizing a precisely targeted individualized circRNA therapeutics. In the future, AI is expected to continue driving end-to-end circRNA lifecycle optimization from the design to the regulation of formulation, supporting the early clinical translation of circRNA therapies.

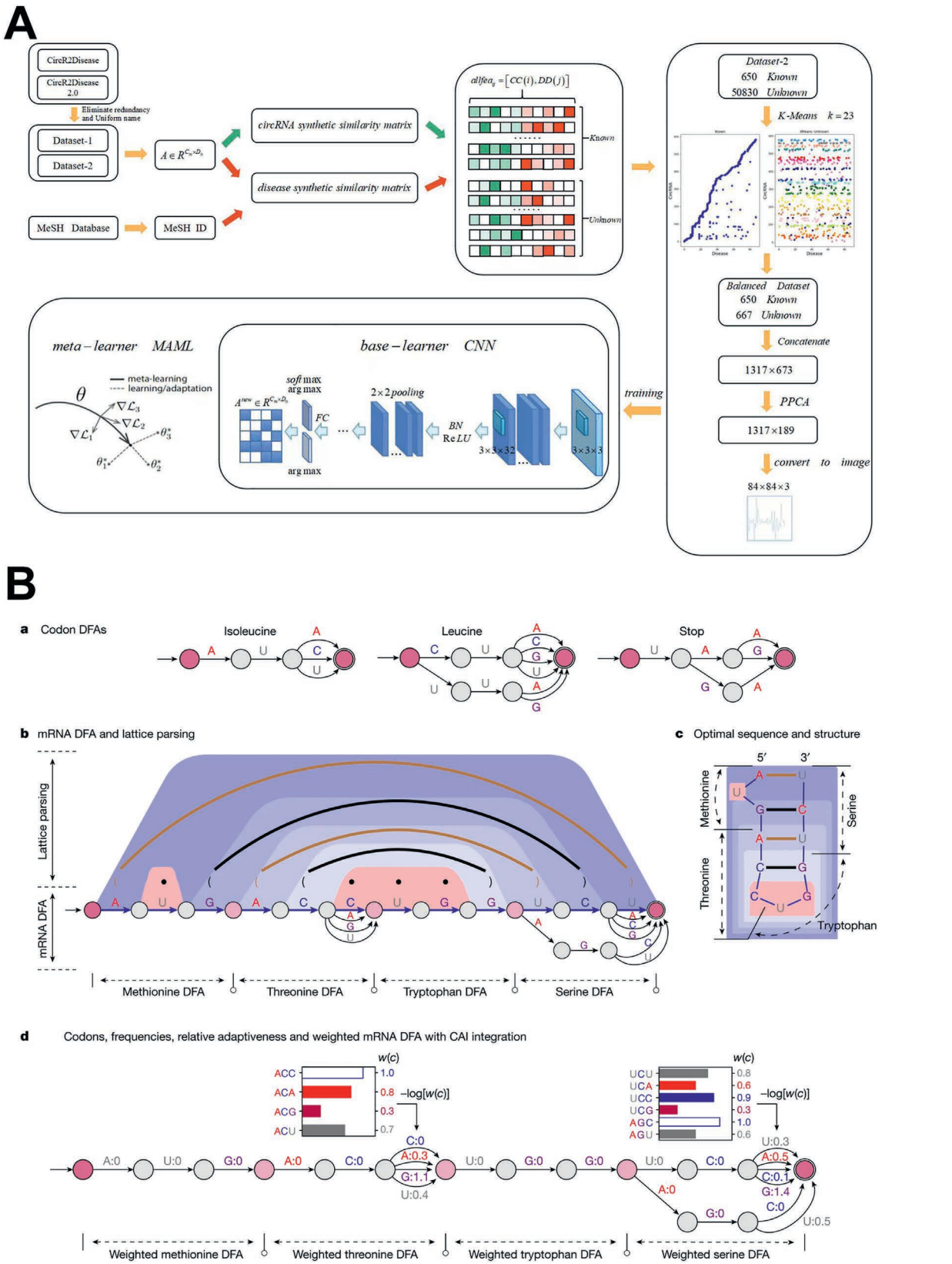

Figure 11 AI-driven optimization and prediction in circRNA research. (A) A meta-learning model for predicting circRNA-disease associations, utilizing model-agnostic meta-learning in conjunction with CNN. Adapted with permission from Ref. 178 © 2024, IEEE. (B) Identification of the optimal RNA sequence based on the Linear Design algorithm. Adapted with permission from Ref. 180 © 2023, The Author(s).

Table 3 Exogenous circRNA in tumor therapy.

circRNA	Delivery vector	Mechanism	Ref.
TI-0093	LNP	Encodes HPV16 E7/E6 to induce immunity (clinical trial)	172
Apt-circRNAs	Subcutaneous injection	Encodes tumor neoantigens (first-in-human trial)	125
acircRNA	Exosome	The aptamer inhibits CTNNB1 and NFKB pathways in BCa cells	115
circRNA^{CAR}	LNP	Primarily activates the vitality of immune cells by encoding CAR	122
circOVA	GSer-CART	Induces a strong T cell response by encoding OVA	170
GSDMD^{ENG} circRNA	LNP	The translated GSDMD leads to mtROS leakage, triggering antitumor immunity; mitochondrial autophagy mediates adaptive immune responses	173
circRERE	AAV	It stabilizes MAVS expression by adsorbing miR-6837-3p, thereby activating the type I IFN signaling pathway	113
circRNA$^{anti-CD19\ CAR}$	Electroporation transfection	Encodes anti-CD19 CAR protein in T cells	101
circRNA 3 × PTPN2	LNP	Encodes the liver cancer-specific tumor neoantigen Ptpn2_I383T	124
cmRNA	Intratumoral injection	Encodes multiple cytokine	126
circ-SHPRH	AAV	Circ-SHPRH induces the expression of P21 by encoding SHPRH-146aa, inhibiting the CDK4-cyclin D complex and blocking the cell cycle progression	117
hsa_circ_0000520	Lentiviruses	Acts as a scaffold to promote the ubiquitination and degradation of LIN28A	174
circFAM53B	Liposome	Encodes peptides with high affinity for HLA-I and HLA-II to enhance infiltration of T cells	69
Circ-arRNA	LNP	Acts for the *in vivo* RNA editing-mediated repair of the *TP53-W53X* nonsense mutation	134
circFOXO3 plasmids	Gold nanoparticles	Induces cell apoptosis	54
circUGP2 plasmids	LNP	Upregulates *ADGRB1* expression by sponging miR-3191-5p and acts as a transcriptional co-activator of PURB	114
circRNA-IL23	LNP	Combines IL-23 and MSA-2-Pt to inhibit tumor	127

AAV, adeno-associated virus; LNP, lipid nanoparticle.

6. Conclusions

Although the exploration of circRNA is still at early stages, the physiological mechanisms that have been elucidated, along with accumulating evidence related to tumor progression, demonstrate significant prospects in therapy (Table 3). circRNA possesses a unique closed circular structure, coupled with the absence of 3′ and 5′ terminal modifications and pathogen recognition sequences, which confers superior stability *in vivo* and reduces degradation during storage, transport, and systemic delivery. CircRNAs are widely expressed in almost all cell types, with a significant portion regulating gene expression by binding to miRNAs and proteins. circRNAs can also produce peptides in a cap-independent mechanism. These intrinsic features have positioned synthetic circRNA as a promising candidate for RNA-based therapeutics. circRNA has demonstrated superior efficacy over linear RNA in various therapeutic areas such as vaccines, protein replacement, and gene editing. The multiple binding sites on circRNA can sustainably interact with miRNA and RBPs, effectively repressing oncogenic pathways. circRNA vaccines yield higher abundance proteins than linear mRNA, inducing stronger immune responses with prolonged immune efficacy. circRNA can also serve as a vector for gene editing to achieve precise knockout of pathogenic genes.

High-throughput screening and AI-assisted design have accelerated the identification of functional circRNA structure. These structures can be synthesized and cyclized *in vitro* using enzymatic ligation, chemical ligation, or ribozyme self-splicing methods. Currently, the PIE is the most widely applied method, producing circular transcripts through rearranging the intron structure, followed by spontaneous splicing within GTP and Mg^{2+} and other reactions. Residual linear precursors, free introns, template DNA, and other byproducts are removed through exonuclease degradation and electrophoresis or chromatography to minimize the non-specific immunogenicity. Nano drug delivery systems have brought promising strategies for the *in vivo* expression of circRNA. Delivery platforms such as LNP, VLP, and exosomes have been employed to encapsulate circRNA due to their good safety, targeting specificity, and stability. The tissue- and cell-targeting potential of circRNA is achieved through structural modifications and surface engineering of these carriers. The LVP-based Tornado system facilitates intracellular self-circularization of circRNA while maintaining high biological activity, expanding the application in circRNA delivery.

Despite these advantages, several barriers still impede the mechanism exploration and clinical transformation, including identifying high-quality targets, scaling industrial circRNA production and purification, improving encapsulation, as well as mitigating immunogenicity of the material itself. Future research will focus more on overcoming these obstacles to make breakthroughs. Firstly, in terms of mechanism exploration, the sheer abundance of circRNAs far exceeds that of upstream genes; thus, it remains challenging to ascertain how tumor circRNAs are dysregulated through genetic inheritance. Genetic alterations, epigenetic modifications, and protein-level perturbations may all contribute to dysregulated circRNA expression[185]. A single circRNA may have multiple competitive binding sites, and the expression profiles vary significantly across different cell types and subcellular localizations, resulting in distinct functionalities. It remains unclear whether modulating circRNAs within a specific pathway may perturb competitive interactions and inadvertently influence unrelated signaling networks. Advances in RNA-Seq, CRISPR-Cas9 editing, and BSJ-specific probes have the potential to minimize false positives in screening differential circRNAs. Emerging nano-omics tools such as nanopore sequencing offer new opportunities to dissect relationships of circRNA and tumors[186]. However, there is still a lack of a gold standard algorithm for quantifying circRNA abundance. Additionally, the lack of a

comprehensive circRNA library further complicates the screening process, hindering the efficient identification[41].

The advancement of circRNA therapeutics is further constrained by the limited efficiency of current preparation strategies. It is essential to first cyclize the long or structurally complex sequence. The incorporation of specific RBP motifs can enhance the efficiency of circularization by promoting the proximity of splice sites. For instance, the introduction of QKI allows for binding to two distant regions of RNA, forming a dimer that brings the two ends closer together[187]. For effective circRNA translation, modifications are often required to obtain sufficient translation levels. Incorporation of translational enhancers, such as Poly(A)-binding protein binding sequence EIF4G, into the 5′ UTR of circRNA can improve the translation efficiency. However, such modifications may result in an excessively long transcript that subsequently reduces cyclization efficiency and increases susceptibility to degradation[188]. Regarding the IRES-dependent translation mechanism, various truncated IRES optimization strategies have consistently led to reduced translation efficiency, as maintaining the stability in the secondary structure of IRES is critical. One feasible solution involves incorporating a series of minimal spacer sequences, which can nearly double cyclization efficiency with only a slight increase in transcript length[188]. Additionally, alternative RNA modifications have emerged as compelling alternatives, such as non-IRES-dependent m^6A modifications that utilize YTHDF3 m^6A readers to recognize m^6A within a short DRACH and initiate translation. Additionally, the m^6A motif can be specifically recognized by YTHDF2 within phase-separated condensates, thereby preventing RIGI exposure and reducing immunogenicity[110]. However, m^6A modification may also induce changes in secondary structure folding, potentially impacting normal function. Furthermore, YTHDF2 is capable of binding to RNase P/MRP *via* HRSP12, which initiates rapid degradation of circRNA. Consequently, further investigation into m^6A and other modifications is warranted[189]. Other motifs, such as a diverse array of 5′ modifications, may reveal additional functionalities. A comprehensive understanding of methylation modifications or capping necessitates further exploration from immunological, stability-related, and efficiency perspectives. In addition, for large molecular proteins like traditional Cas9 (>3000 nt), Compact alternatives, such as Cas12a with its smaller size, low off-target effect, and A/T-rich regions preference, represent an alternative solution[130]. Simultaneously, it is crucial to seek an alternative circularization method to achieve the desired length and structure. Researchers have also promoted circularization by design, circRNA-promoting RNA (cpRNA) to increase the stable pairing of the flanking introns by bringing the terminal sequences of precursor RNA closer together[40]. At the manufacturing level, purification remains a major unmet need. Various purification techniques, such as HPLC and electrophoresis, can improve the resolution of products; they are currently limited to small-scale laboratory applications, leaving industrial-scale purification technology still under development. Another major challenge in preparation is immunogenicity. Although the immunostimulatory effect of circRNA is considerably lower compared to the corresponding linear RNA, Chen et al.[190] have shown that circRNA spliced with the intron from the T4 phage td gene can induce the expression of RIGI, while endogenous introns appear to mitigate this effect. Some researchers have also proposed that RNA modification may regulate immune gene expression, but there is currently no standardized experimental protocol for verifying immunogenicity[88]. In addition, studies have explored leveraging the immunogenic properties of circRNA by delivering it as an immune adjuvant in conjunction with soluble proteins to elicit specific immune responses[191].

The delivery of circRNA remains a critical issue. Although the stability of circRNA is better than that of linear RNA, it still requires an appropriate carrier to prevent nonspecific degradation and ensure bioavailability. Active targeting modifications are particularly important, especially in the high osmotic pressure environment of solid tumors. LNP, VLP, and exosomes demonstrate excellent biocompatibility and structural stability. Further improvements are needed in material modification and loading capabilities. Enhancing loading efficiency must be balanced against risks such as particle aggregation, elevated immunogenicity, and other side effects that may arise from alterations in parameters such as Zeta and particle size. Overcoming circRNA inactivation presents another significant obstacle. While delivery-associated immunogenicity has received increasing attention, a comprehensive and systematic safety assessment remains insufficient. In addition to adverse reactions stemming from residual impurities, potential off-target effects and immune inflammation induced by repeated dosing necessitate further investigation. Intravenous carriers tend to accumulate in the liver, with this accumulation exacerbated by high doses or chronic administration[192]. Chronic toxicity to non-target organs such as the kidneys and lungs, alongside the liver, also warrants additional scrutiny. Immune responses or neutralizing antibodies elicited by components of the carrier itself can result in unexpected immune reactions and may alter existing immune recognition, thereby diminishing efficacy[193,194]. Furthermore, different administration routes may lead to distinct profiles of innate immune activation. A more detailed quantitative analysis of pharmacokinetics, biodistribution analyses to define delivery, clearance, and tissue-specific accumulation is essential within biological systems. The functional degradation effects associated with industrial storage and transportation of carriers also require ongoing clarification. For viral vectors specifically, potential genome insertion represents the most critical concern. Employing non-integrating viral particles has substantially mitigated this risk; additionally, mechanisms such as integration site monitoring are utilized to further reduce this concern. Concurrently, the development of novel carrier systems remains an important direction. For example, the Selective Endogenous eNcapsidation for cellular Delivery system utilizes a mechanism involving certain retrotransposon-derived proteins in mammalian genomes, which can form capsid particles that encapsulate their own mRNA for transfer *via* vesicles. The PEG10 protein derived from human retrotransposon factors was selected and modified to encapsulate the target specifically UTR sequence modified with PEG10 at both ends by reprogramming PEG10-VLP. The specificity of cellular targeting was further enhanced through fusing targeting peptides, facilitating the successful delivery and expression of the CRISPR-Cas9 *in vivo*. Theoretically, the same mechanism could be applied to circRNA or its precursor sequences through UTR reprogramming[195]. In addition, achieving efficient overexpression of circRNA *in vivo* remains an urgent challenge that must be solved. The transposon transfection method previously discussed offers a feasible strategy that utilizes specific drivers to enable controlled expression in targeted cells. However, this approach also requires the delivery capabilities of an effective vector.

CircRNA-based therapy presents an unprecedented method for precision treatment. Several diagnostic clinical trials are underway to investigate circRNA dysregulation. Concurrently, clinical trials

focusing on exogenous circRNA delivery have begun to advance. For example, TI-0093 is currently undergoing Phase I clinical trials for advanced recurrent or metastatic solid tumors associated with $HPV16^{+}$, which involves five intramuscular injections aimed at evaluating safety and early efficacy. Additionally, the multivalent neoantigen circRNA vaccine apt-circRNA-KR2 has already shown preliminary safety and immune responsiveness in first-in-human trials[125,172]. Beyond oncology, exogenous circRNA therapeutics are being explored for ischemic heart failure and radiation-induced xerostomia. It is evident that with deeper insights into the structural biology, intracellular dynamics, and regulatory mechanisms of circRNA-coupled with advancements in circRNA production, purification, and delivery technologies. This innovative RNA therapeutic approach holds considerable promise for application in oncology and potentially facilitates precise tumor treatment.

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Author contributions

Xu Shi and Yue Zhao and Yunkai Tang contributed to the data collection and organization, writing-original draft preparation, figure and table drawing. Gang Fu and Chen Qian and Feng Li collected and provided part of the literature data. Zheyu Yang and Wenguo Cui contributed to the manuscript proofreading and correction. Wei Cai contributed to determining the general direction and objectives of the research, and revision of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2024;**74**:229−63.
2. Dutta R, Mahato RI. Recent advances in hepatocellular carcinoma therapy. *Pharmacol Ther* 2017;**173**:106−17.
3. Mansouri V, Beheshtizadeh N, Gharibshahian M, Sabouri L, Varzandeh M, Rezaei N. Recent advances in regenerative medicine strategies for cancer treatment. *Biomed Pharmacother* 2021;**141**: 111875.
4. Alberer M, Gnad-Vogt U, Hong HS, Mehr KT, Backert L, Finak G, et al. Safety and immunogenicity of a mRNA rabies vaccine in healthy adults: an open-label, non-randomised, prospective, first-in-human phase 1 clinical trial. *Lancet* 2017;**390**:1511−20.
5. An D, Schneller JL, Frassetto A, Liang S, Zhu X, Park JS, et al. Systemic messenger RNA therapy as a treatment for methylmalonic acidemia. *Cell Rep* 2017;**21**:3548−58.
6. Crooke ST, Witztum JL, Bennett CF, Baker BF. RNA-targeted therapeutics. *Cell Metab* 2019;**29**:501.
7. Wang F, Zuroske T, Watts JK. RNA therapeutics on the rise. *Nat Rev Drug Discov* 2020;**19**:441−2.
8. Zhu Y, Zhu L, Wang X, Jin H. RNA-based therapeutics: an overview and prospectus. *Cell Death Dis* 2022;**13**:644.
9. Zamecnik PC, Stephenson ML. Inhibition of *Rous sarcoma* virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci U S A* 1978;**75**:280−4.
10. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med* 2018;**379**:11−21.
11. Roehr B. Fomivirsen approved for CMV retinitis. *J Int Assoc Phys AIDS Care* 1998;**4**:14−6.
12. Çakan E, Lara OD, Szymanowska A, Bayraktar E, Chavez-Reyes A, Lopez-Berestein G, et al. Therapeutic antisense oligonucleotides in oncology: from bench to bedside. *Cancers (Basel)* 2024;**16**:2940.
13. Shanaa OA, Rumyantsev A, Sambuk E, Padkina M. *In vivo* production of RNA aptamers and nanoparticles: problems and prospects. *Molecules* 2021;**26**:1422.
14. Kim YK. RNA therapy: rich history, various applications and unlimited future prospects. *Exp Mol Med* 2022;**54**:455−65.
15. Zhang C, Zhang B. RNA therapeutics: updates and future potential. *Sci China Life Sci* 2023;**66**:12−30.
16. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. *Physiol Rev* 2016;**96**:1297−325.
17. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N Engl J Med* 2021;**384**:403−16.
18. Kauffman KJ, Webber MJ, Anderson DG. Materials for non-viral intracellular delivery of messenger RNA therapeutics. *J Control Release* 2016;**240**:227−34.
19. Kim HY, Kim TR, Kim SH, Kim IH, Ko Y, Yun S, et al. Genotoxicity evaluation of self-assembled-micelle inhibitory RNA-targeting amphiregulin (SAMiRNA-AREG), a novel siRNA nanoparticle for the treatment of fibrotic disease. *Drug Chem Toxicol* 2022;**45**: 2109−15.
20. Dalpke A, Helm M. RNA mediated toll-like receptor stimulation in health and disease. *RNA Biol* 2012;**9**:828−42.
21. Patop IL, Wust S, Kadener S. Past, present, and future of circRNAs. *EMBO J* 2019;**38**:e100836.
22. Wu W, Ji P, Zhao F. CircAtlas: an integrated resource of one million highly accurate circular RNAs from 1070 vertebrate transcriptomes. *Genome Biol* 2020;**21**:101.
23. Rybak-Wolf A, Stottmeister C, Glazar P, Jens M, Pino N, Giusti S, et al. Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol Cell* 2015;**58**:870−85.
24. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. *PLoS Genet* 2013;**9**: e1003777.
25. Hama Faraj GS, Hussen BM, Abdullah SR, Fatih Rasul M, Hajiesmaeili Y, Baniahmad A, et al. Advanced approaches of the use of circRNAs as a replacement for cancer therapy. *Noncoding RNA Res* 2024;**9**:811−30.
26. Zang X, Jiang J, Gu J, Chen Y, Wang M, Zhang Y, et al. Circular RNA EIF4G3 suppresses gastric cancer progression through inhibition of β-catenin by promoting δ-catenin ubiquitin degradation and upregulating SIK1. *Mol Cancer* 2022;**21**:141.
27. Pamudurti NR, Bartok O, Jens M, Ashwal-Fluss R, Stottmeister C, Ruhe L, et al. Translation of circRNAs. *Mol Cell* 2017;**66**:9−21 e7.
28. Kristensen LS, Jakobsen T, Hager H, Kjems J. The emerging roles of circRNAs in cancer and oncology. *Nat Rev Clin Oncol* 2022;**19**: 188−206.
29. Wang Y, Wang Z. Efficient backsplicing produces translatable circular mRNAs. *RNA* 2015;**21**:172−9.
30. Xu C, Zhang J. Mammalian circular RNAs result largely from splicing errors. *Cell Rep* 2021;**36**:109439.
31. Enuka Y, Lauriola M, Feldman ME, Sas-Chen A, Ulitsky I, Yarden Y. Circular RNAs are long-lived and display only minimal early

alterations in response to a growth factor. *Nucleic Acids Res* 2016;**44**: 1370−83.
32. Hussen BM, Abdullah SR, Hama Faraj GS, Rasul MF, Salihi A, Ghafouri-Fard S, et al. Exosomal circular RNA: a signature for lung cancer progression. *Cancer Cell Int* 2022;**22**:378.
33. Wesselhoeft RA, Kowalski PS, Parker-Hale FC, Huang Y, Bisaria N, Anderson DG. RNA circularization diminishes immunogenicity and can extend translation duration *in vivo*. *Mol Cell* 2019;**74**:508−520 e4.
34. Zhang XO, Wang HB, Zhang Y, Lu X, Chen LL, Yang L. Complementary sequence-mediated exon circularization. *Cell* 2014;**159**: 134−47.
35. Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, et al. Exon-intron circular RNAs regulate transcription in the nucleus. *Nat Struct Mol Biol* 2015;**22**:256−64.
36. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 2013;**19**:141−57.
37. Konarska MM, Grabowski PJ, Padgett RA, Sharp PA. Characterization of the branch site in lariat RNAs produced by splicing of mRNA precursors. *Nature* 1985;**313**:552−7.
38. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. *Nat Biotechnol* 2014;**32**:453−61.
39. Khan MA, Reckman YJ, Aufiero S, van den Hoogenhof MM, van der Made I, Beqqali A, et al. RBM20 regulates circular RNA production from the titin gene. *Circ Res* 2016;**119**:996−1003.
40. He Z, Ji H, Xia B, Cao X, Huang Y, Zhu Q. Invention of circRNA promoting RNA to specifically promote circRNA production. *Nucleic Acids Res* 2024;**52**:e83.
41. Nielsen AF, Bindereif A, Bozzoni I, Hanan M, Hansen TB, Irimia M, et al. Best practice standards for circular RNA research. *Nat Methods* 2022;**19**:1208−20.
42. Vo JN, Cieslik M, Zhang Y, Shukla S, Xiao L, Zhang Y, et al. The landscape of circular RNA in cancer. *Cell* 2019;**176**. 869-81 e13.
43. Kristensen LS, Hansen TB, Venø MT, Kjems J. Circular RNAs in cancer: opportunities and challenges in the field. *Oncogene* 2018;**37**: 555−65.
44. Artemaki PI, Scorilas A, Kontos CK. Circular RNAs: a new piece in the colorectal cancer puzzle. *Cancers (Basel)* 2020;**12**:2464.
45. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, et al. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013;**495**:384−8.
46. Hansen TB, Kjems J, Damgaard CK. Circular RNA and miR-7 in cancer. *Cancer Res* 2013;**73**:5609−12.
47. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 2013;**495**:333−8.
48. Pan H, Li T, Jiang Y, Pan C, Ding Y, Huang Z, et al. Overexpression of circular RNA ciRS-7 abrogates the tumor suppressive effect of miR-7 on gastric cancer *via* PTEN/PI3K/AKT signaling pathway. *J Cell Biochem* 2018;**119**:440−6.
49. Zhang L. Circular RNA: the main regulator of energy metabolic reprogramming in cancer cells. *Thorac Cancer* 2020;**11**:6−7.
50. Hansen TB, Wiklund ED, Bramsen JB, Villadsen SB, Statham AL, Clark SJ, et al. miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *EMBO J* 2011;**30**: 4414−22.
51. Han K, Wang FW, Cao CH, Ling H, Chen JW, Chen RX, et al. CircLONP2 enhances colorectal carcinoma invasion and metastasis through modulating the maturation and exosomal dissemination of microRNA-17. *Mol Cancer* 2020;**19**:60.
52. Schreiner S, Didio A, Hung LH, Bindereif A. Design and application of circular RNAs with protein-sponge function. *Nucleic Acids Res* 2020;**48**:12326−35.
53. Du WW, Yang W, Liu E, Yang Z, Dhaliwal P, Yang BB. Foxo3 circular RNA retards cell cycle progression *via* forming ternary complexes with p21 and CDK2. *Nucleic Acids Res* 2016;**44**: 2846−58.
54. Du WW, Fang L, Yang W, Wu N, Awan FM, Yang Z, et al. Induction of tumor apoptosis through a circular RNA enhancing Foxo3 activity. *Cell Death Differ* 2017;**24**:357−70.
55. Chen D, Zeng S, Qiu H, Yang M, Lin X, Lv X, et al. Circ-FOXO3 inhibits triple-negative breast cancer growth and metastasis *via* regulating WHSC1−H3K36me2−Zeb2 axis. *Cell Signal* 2024;**117**:111079.
56. Li H, Feng H, Zhang T, Wu J, Shen X, Xu S, et al. CircHAS2 activates CCNE2 to promote cell proliferation and sensitizes the response of colorectal cancer to anlotinib. *Mol Cancer* 2024;**23**:59.
57. Wang X, Chen T, Li C, Li W, Zhou X, Li Y, et al. CircRNA-CREIT inhibits stress granule assembly and overcomes doxorubicin resistance in TNBC by destabilizing PKR. *J Hematol Oncol* 2022;**15**:122.
58. Meng L, Zhang Y, Wu P, Li D, Lu Y, Shen P, et al. CircSTX6 promotes pancreatic ductal adenocarcinoma progression by sponging miR-449b-5p and interacting with CUL2. *Mol Cancer* 2022;**21**:121.
59. Legnini I, Di Timoteo G, Rossi F, Morlando M, Briganti F, Sthandier O, et al. Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. *Mol Cell* 2017;**66**:22−37.e9.
60. Chen CK, Cheng R, Demeter J, Chen J, Weingarten-Gabbay S, Jiang L, et al. Structured elements drive extensive circular RNA translation. *Mol Cell* 2021;**81**:4300−4318 e13.
61. Hwang HJ, Kim YK. Molecular mechanisms of circular RNA translation. *Exp Mol Med* 2024;**56**:1272−80.
62. Wang S, Latallo MJ, Zhang Z, Huang B, Bobrovnikov DG, Dong D, et al. Nuclear export and translation of circular repeat-containing intronic RNA in C9ORF72-ALS/FTD. *Nat Commun* 2021;**12**:4908.
63. Yang Y, Fan X, Mao M, Song X, Wu P, Zhang Y, et al. Extensive translation of circular RNAs driven by *N*(6)-methyladenosine. *Cell Res* 2017;**27**:626−41.
64. Lu J, Ru J, Chen Y, Ling Z, Liu H, Ding B, et al. *N*(6)-methyladenosine-modified circSTX6 promotes hepatocellular carcinoma progression by regulating the HNRNPD/ATF3 axis and encoding a 144 amino acid polypeptide. *Clin Transl Med* 2023;**13**:e1451.
65. Mohsen MG, Kool ET. The discovery of rolling circle amplification and rolling circle transcription. *Acc Chem Res* 2016;**49**:2540−50.
66. Abe N, Matsumoto K, Nishihara M, Nakano Y, Shibata A, Maruyama H, et al. Rolling circle translation of circular RNA in living human cells. *Sci Rep* 2015;**5**:16435.
67. Wang X, Xuan Y, Han Y, Ding X, Ye K, Yang F, et al. Regulation of HIV-1 Gag-Pol expression by shiftless, an inhibitor of programmed-1 ribosomal frameshifting. *Cell* 2019;**176**:625−35 e14.
68. Song R, Ma S, Xu J, Ren X, Guo P, Liu H, et al. A novel polypeptide encoded by the circular RNA ZKSCAN1 suppresses HCC *via* degradation of mTOR. *Mol Cancer* 2023;**22**:16.
69. Huang D, Zhu X, Ye S, Zhang J, Liao J, Zhang N, et al. Tumour circular RNAs elicit anti-tumour immunity by encoding cryptic peptides. *Nature* 2024;**625**:593−602.
70. Fathima A, Ameer SF, Kerzabi RI, Giordo R, Nasrallah GK, Zayed H, et al. Natural antioxidants as regulators of circular RNA expression and function. *Wiley Interdiscip Rev RNA* 2025;**16**:e70023.
71. Chen D, Chou FJ, Chen Y, Tian H, Wang Y, You B, et al. Targeting the radiation-induced TR4 nuclear receptor-mediated QKI/circZEB1/miR-141-3p/ZEB1 signaling increases prostate cancer radiosensitivity. *Cancer Lett* 2020;**495**:100−11.
72. Moser JC, Papadopoulos KP, Ahnert JR, Ofir-Rosenfeld Y, Holz JB. Phase 1 dose escalation and cohort expansion study evaluating safety, PK, PD and clinical activity of STC-15, a METTL-3 inhibitor, in patients with advanced malignancies. *J Clin Oncol* 2024;**42**:2586.
73. Li X, Yang L, Chen LL. The biogenesis, functions, and challenges of circular RNAs. *Mol Cell* 2018;**71**:428−42.
74. Petkovic S, Müller S. Synthesis and engineering of circular RNAs. *Methods Mol Biol* 2018;**1724**:167−80.
75. Obi P, Chen YG. The design and synthesis of circular RNAs. *Methods* 2021;**196**:85−103.
76. Chen X, Lu Y. Circular RNA: biosynthesis *in vitro*. *Front Bioeng Biotechnol* 2021;**9**:787881.
77. Fantoni NZ, El-Sagheer AH, Brown T. A Hitchhiker's guide to click-chemistry with nucleic acids. *Chem Rev* 2021;**121**:7122−54.

78. Moore MJ. Joining RNA molecules with T4 DNA ligase. *Methods Mol Biol* 1999;**118**:11−9.
79. Lee KH, Kim S, Lee SW. Pros and cons of *in vitro* methods for circular RNA preparation. *Int J Mol Sci* 2022;**23**:13247.
80. Kim H, Kim D, Moon S, Lee JB. Efficient circular RNA synthesis through Gap-DNA splint-mediated ligation. *Nanoscale* 2024;**16**: 15529−32.
81. Li S, Chu Y, Guo X, Mao C, Xiao SJ. Circular RNA oligonucleotides: enzymatic synthesis and scaffolding for nanoconstruction. *Nanoscale Horiz* 2024;**9**:1749−55.
82. Bullard DR, Bowater RP. Direct comparison of nick-joining activity of the nucleic acid ligases from bacteriophage T4. *Biochem J* 2006; **398**:135−44.
83. Petkovic S, Muller S. RNA circularization strategies *in vivo* and *in vitro*. *Nucleic Acids Res* 2015;**43**:2454−65.
84. Liu X, Abraham JM, Cheng Y, Wang Z, Wang Z, Zhang G, et al. Synthetic circular RNA functions as a miR-21 sponge to suppress gastric carcinoma cell proliferation. *Mol Ther Nucleic Acids* 2018;**13**: 312−21.
85. Nandakumar J, Ho CK, Lima CD, Shuman S. RNA substrate specificity and structure-guided mutational analysis of bacteriophage T4 RNA ligase 2. *J Biol Chem* 2004;**279**:31337−47.
86. Cheng K, An R, Cui Y, Zhang Y, Han X, Sui Z, et al. RNA ligation of very small pseudo nick structures by T4 RNA ligase 2, leading to efficient production of versatile RNA rings. *RSC Adv* 2019;**9**: 8620−7.
87. Chen H, Cheng K, Liu X, An R, Komiyama M, Liang X. Preferential production of RNA rings by T4 RNA ligase 2 without any splint through rational design of precursor strand. *Nucleic Acids Res* 2020; **48**:e54.
88. Liu C-X, Guo S-K, Nan F, Xu Y-F, Yang L, Chen L-L. RNA circles with minimized immunogenicity as potent PKR inhibitors. *Mol Cell* 2022;**82**. 420-34.e6.
89. Puttaraju M, Been MD. Group I permuted intron-exon (PIE) sequences self-splice to produce circular exons. *Nucleic Acids Res* 1992;**20**:5357−64.
90. Wesselhoeft RA, Kowalski PS, Anderson DG. Engineering circular RNA for potent and stable translation in eukaryotic cells. *Nat Commun* 2018;**9**:2629.
91. Qi S, Wang H, Liu G, Qin Q, Gao P, Ying B. Efficient circularization of protein-encoding RNAs *via* a novel cis-splicing system. *Nucleic Acids Res* 2024;**52**:10400−15.
92. Shen Y, Li B, Dong L, Tang W, Ren J, Chen F, et al. Self-splicing RNA circularization facilitated by intact group I and II introns. *Nat Commun* 2025;**16**:7376.
93. Rausch JW, Heinz WF, Payea MJ, Sherpa C, Gorospe M, Le Grice SFJ. Characterizing and circumventing sequence restrictions for synthesis of circular RNA *in vitro*. *Nucleic Acids Res* 2021;**49**: e35.
94. Lee KH, Kim S, Song J, Han SR, Kim JH, Lee SW. Efficient circular RNA engineering by end-to-end self-targeting and splicing reaction using Tetrahymena group I intron ribozyme. *Mol Ther Nucleic Acids* 2023;**33**:587−98.
95. Mikheeva S, Hakim-Zargar M, Carlson D, Jarrell K. Use of an engineered ribozyme to produce a circular human exon. *Nucleic Acids Res* 1997;**25**:5085−94.
96. Niu D, Wu Y, Lian J. Circular RNA vaccine in disease prevention and treatment. *Signal Transduct Targeted Ther* 2023;**8**:341.
97. Toor N, Keating KS, Taylor SD, Pyle AM. Crystal structure of a self-spliced group II intron. *Science* 2008;**320**:77−82.
98. Pyle AM. The tertiary structure of group II introns: implications for biological function and evolution. *Crit Rev Biochem Mol Biol* 2010; **45**:215−32.
99. Jarrell KA. Inverse splicing of a group II intron. *Proc Natl Acad Sci U S A* 1993;**90**:8624−7.
100. Murray HL, Mikheeva S, Coljee VW, Turczyk BM, Donahue WF, Bar-Shalom A, et al. Excision of group II introns as circles. *Mol Cell* 2001;**8**:201−11.
101. Hu Q, Zhao H, Zhou K, Tian X, Wang Q, Hua X, et al. Scarless circular mRNA-based CAR-T cell therapy elicits superior antitumor efficacy. *Signal Transduct Targeted Ther* 2025;**10**:411.
102. Branch AD, Robertson HD. A replication cycle for viroids and other small infectious RNA's. *Science* 1984;**223**:450−5.
103. Luan X, Wang L, Song G, Zhou W. Innate immune responses to RNA: sensing and signaling. *Front Immunol* 2024;**15**:1287940.
104. Loan Young T, Chang Wang K, James Varley A, Li B. Clinical delivery of circular RNA: lessons learned from RNA drug development. *Adv Drug Deliv Rev* 2023;**197**:114826.
105. Vincent HA, Deutscher MP. Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem* 2006;**281**:29769−75.
106. Vincent HA, Deutscher MP. Insights into how RNase R degrades structured RNA: analysis of the nuclease domain. *J Mol Biol* 2009; **387**:570−83.
107. Chursov A, Kopetzky SJ, Bocharov G, Frishman D, Shneider A. RNAtips: analysis of temperature-induced changes of RNA secondary structure. *Nucleic Acids Res* 2013;**41**:W486−91.
108. Zhang Z, Li W, Ren X, Luo D, Yuan X, Yu L, et al. Mitigating cellular dysfunction through contaminant reduction in synthetic circRNA for high-efficiency mRNA-based cell reprogramming. *Adv Sci (Weinh)* 2025;**12**:e2416629.
109. Guillen-Cuevas K, Lu X, Birtwistle MR, Husson SM. Purifying circular RNA by ultrafiltration. *Sep Purif Technol* 2025;**366**:132809.
110. Chen YG, Chen R, Ahmad S, Verma R, Kasturi SP, Amaya L, et al. *N*6-methyladenosine modification controls circular RNA immunity. *Mol Cell* 2019;**76**:96−109 e9.
111. Fan HY, Zhao MD, Jiang HJ, Yu ZW, Fan YJ, Liang XH, et al. Cisplatin-based miRNA delivery strategy inspired by the circCPNE1/miR-330-3p pathway for oral squamous cell carcinoma. *Acta Pharm Sin B* 2024;**14**:2748−60.
112. Geng Y, Zheng X, Hu W, Wang Q, Xu Y, He W, et al. Hsa_circ_0009361 acts as the sponge of miR-582 to suppress colorectal cancer progression by regulating APC2 expression. *Clin Sci (Lond)* 2019;**133**:1197−213.
113. Ding N, You AB, Yang H, Hu GS, Lai CP, Liu W, et al. A tumor-suppressive molecular axis EP300/circRERE/miR-6837-3p/MAVS activates type I IFN pathway and antitumor immunity to suppress colorectal cancer. *Clin Cancer Res* 2023;**29**:2095−109.
114. Chen RX, Liu SC, Kan XC, Wang YR, Wang JF, Wang TL, et al. CircUGP2 suppresses intrahepatic cholangiocarcinoma progression *via* p53 signaling through interacting with PURB to regulate ADGRB1 transcription and sponging miR-3191-5p. *Adv Sci (Weinh)* 2024;**11**:e2402329.
115. Zhou Q, Fang L, Tang Y, Wang Q, Tang X, Zhu L, et al. Exosome-mediated delivery of artificial circular RNAs for gene therapy of bladder cancer. *J Cancer* 2024;**15**:1770−8.
116. Unti MJ, Jaffrey SR. Highly efficient cellular expression of circular mRNA enables prolonged protein expression. *Cell Chem Biol* 2024; **31**:163−176.e5.
117. Chang S, Ren D, Zhang L, Liu S, Yang W, Cheng H, et al. Therapeutic SHPRH-146aa encoded by circ-SHPRH dynamically upregulates P21 to inhibit CDKs in neuroblastoma. *Cancer Lett* 2024; **598**:217120.
118. Zeng C, Zhang C, Walker PG, Dong Y. Formulation and delivery technologies for mRNA vaccines. *Curr Top Microbiol Immunol* 2022; **440**:71−110.
119. Oude Blenke E, Ornskov E, Schoneich C, Nilsson GA, Volkin DB, Mastrobattista E, et al. The storage and in-use stability of mRNA vaccines and therapeutics: not a cold case. *J Pharmacol Sci* 2023; **112**:386−403.
120. Chen H, Liu D, Aditham A, Guo J, Huang J, Kostas F, et al. Chemical and topological design of multicapped mRNA and capped circular RNA to augment translation. *Nat Biotechnol* 2025;**43**: 1128−43.
121. Su CI, Chuang ZS, Shie CT, Wang HI, Kao YT, Yu CY. A *cis*-acting ligase ribozyme generates circular RNA *in vitro* for ectopic protein functioning. *Nat Commun* 2024;**15**:6607.

122. Wang Y, Lin L, Wang X, Li J, Pan Q, Kou H, et al. Synergically enhanced anti-tumor immunity of *in vivo* panCAR by circRNA vaccine boosting. *Cell Rep Med* 2025;**6**:102250.
123. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Science* 2018;**362**:eaar3593.
124. Wang F, Cai G, Wang Y, Zhuang Q, Cai Z, Li Y, et al. Circular RNA-based neoantigen vaccine for hepatocellular carcinoma immunotherapy. *MedComm* 2024;**5**:e667.
125. Zhang Y, Wang W, Qian X, Chen J, Ding D, Gan S, et al. Programmable aptamer-embedded circular RNAs for targeted antigen-presenting cells immunotherapy. *bioRxiv* 2025. Available from: https://doi.org/10.1101/2025.09.28.679023.
126. Yang J, Zhu J, Sun J, Chen Y, Du Y, Tan Y, et al. Intratumoral delivered novel circular mRNA encoding cytokines for immune modulation and cancer therapy. *Mol Ther Nucleic Acids* 2022;**30**: 184−97.
127. He T, Li Y, Li W, Zhang M, Wang G, Zhou P, et al. Enhanced antitumor efficacy of STING agonist MSA-2 by lipid nanoparticles delivering circular IL-23 mRNA and platinum-modified MSA-2 combination. *Mater Today Bio* 2025;**30**: 101446.
128. Liu L, Li W, Li J, Zhao D, Li S, Jiang G, et al. Circular guide RNA for improved stability and CRISPR-Cas9 editing efficiency *in vitro* and in bacteria. *ACS Synth Biol* 2023;**12**:350−9.
129. Zhang X, Wang X, Lv J, Huang H, Wang J, Zhuo M, et al. Engineered circular guide RNAs boost CRISPR/Cas12a- and CRISPR/Cas13d-based DNA and RNA editing. *Genome Biol* 2023; **24**:145.
130. Liang R, He Z, Zhao KT, Zhu H, Hu J, Liu G, et al. Prime editing using CRISPR-Cas12a and circular RNAs in human cells. *Nat Biotechnol* 2024;**42**:1867−75.
131. Wang Q, Li X, Qi R, Billiar T. RNA editing, ADAR1, and the innate immune response. *Genes* 2017;**8**:41.
132. Nishikura K. Functions and regulation of RNA editing by ADAR deaminases. *Annu Rev Biochem* 2010;**79**:321−49.
133. Katrekar D, Yen J, Xiang Y, Saha A, Meluzzi D, Savva Y, et al. Efficient *in vitro* and *in vivo* RNA editing *via* recruitment of endogenous ADARs using circular guide RNAs. *Nat Biotechnol* 2022;**40**:938−45.
134. Wang J, Zhang W, Li S, Shi W, Li B, Zhang J, et al. RNA editing-mediated correction of TP53 nonsense mutations *via* lipid nanoparticle-delivered circular ADAR-recruiting RNAs. *J Am Chem Soc* 2025;**147**:18512−23.
135. Gumireddy K, Li A, Kossenkov AV, Sakurai M, Yan J, Li Y, et al. The mRNA-edited form of GABRA3 suppresses GABRA3-mediated Akt activation and breast cancer metastasis. *Nat Commun* 2016;**7**: 10715.
136. Wong TL, Loh JJ, Lu S, Yan HHN, Siu HC, Xi R, et al. ADAR1-mediated RNA editing of SCD1 drives drug resistance and self-renewal in gastric cancer. *Nat Commun* 2023;**14**:2861.
137. Huang X, Ma Y, Ma G, Xia Y. Unlocking the therapeutic applicability of LNP-mRNA: chemistry, formulation, and clinical strategies. *Research* 2024;**7**:370.
138. Swingle KL, Hamilton AG, Mitchell MJ. Lipid nanoparticle-mediated delivery of mRNA therapeutics and vaccines. *Trends Mol Med* 2021;**27**:616−7.
139. Rhym LH, Anderson DG. Nanoscale delivery platforms for RNA therapeutics: challenges and the current state of the art. *Med* 2022;**3**: 167−87.
140. Golubovic A, Tsai S, Li B. Bioinspired lipid nanocarriers for RNA delivery. *ACS Bio Med Chem Au* 2023;**3**:114−36.
141. Geng C, Zhou K, Yan Y, Li C, Ni B, Liu J, et al. A preparation method for mRNA-LNPs with improved properties. *J Control Release* 2023;**364**:632−43.
142. Bai X, Chen Q, Li F, Teng Y, Tang M, Huang J, et al. Optimized inhaled LNP formulation for enhanced treatment of idiopathic pulmonary fibrosis *via* mRNA-mediated antibody therapy. *Nat Commun* 2024;**15**:6844.
143. Belliveau NM, Huft J, Lin PJ, Chen S, Leung AK, Leaver TJ, et al. Microfluidic synthesis of highly potent limit-size lipid nanoparticles for *in vivo* delivery of siRNA. *Mol Ther Nucleic Acids* 2012;**1**:e37.
144. Xu S, Xu Y, Solek NC, Chen J, Gong F, Varley AJ, et al. Tumor-tailored ionizable lipid nanoparticles facilitate IL-12 circular RNA delivery for enhanced lung cancer immunotherapy. *Adv Mater* 2024; **36**:e2400307.
145. Kang DD, Hou X, Wang L, Xue Y, Li H, Zhong Y, et al. Engineering LNPs with polysarcosine lipids for mRNA delivery. *Bioact Mater* 2024;**37**:86−93.
146. Muramatsu H, Lam K, Bajusz C, Laczko D, Kariko K, Schreiner P, et al. Lyophilization provides long-term stability for a lipid nanoparticle-formulated, nucleoside-modified mRNA vaccine. *Mol Ther* 2022;**30**:1941−51.
147. Wan J, Yang J, Wang Z, Shen R, Zhang C, Wu Y, et al. A single immunization with core−shell structured lipopolyplex mRNA vaccine against rabies induces potent humoral immunity in mice and dogs. *Emerg Microbes Infect* 2023;**12**:2270081.
148. Wang X, Liu S, Sun Y, Yu X, Lee SM, Cheng Q, et al. Preparation of selective organ-targeting (SORT) lipid nanoparticles (LNPs) using multiple technical methods for tissue-specific mRNA delivery. *Nat Protoc* 2023;**18**:265−91.
149. Huang G, Zheng W, Zhou Y, Wan M, Hu T. Recent advances to address challenges in extracellular vesicle-based applications for lung cancer. *Acta Pharm Sin B* 2024;**14**:3855−75.
150. Ferguson SW, Nguyen J. Exosomes as therapeutics: the implications of molecular composition and exosomal heterogeneity. *J Control Release* 2016;**228**:179−90.
151. Fanale D, Taverna S, Russo A, Bazan V. Circular RNA in exosomes. *Adv Exp Med Biol* 2018;**1087**:109−17.
152. Li Y, Zheng Q, Bao C, Li S, Guo W, Zhao J, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res* 2015;**25**:981−4.
153. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature* 2015;**527**:329−35.
154. Pan Z, Zhao R, Li B, Qi Y, Qiu W, Guo Q, et al. EWSR1-induced circNEIL3 promotes glioma progression and exosome-mediated macrophage immunosuppressive polarization *via* stabilizing IGF2BP3. *Mol Cancer* 2022;**21**:16.
155. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;**29**:341−5.
156. Kurian TK, Banik S, Gopal D, Chakrabarti S, Mazumder N. Elucidating methods for isolation and quantification of exosomes: a review. *Mol Biotechnol* 2021;**63**:249−66.
157. Fan L, Yao L, Li Z, Wan Z, Sun W, Qiu S, et al. Exosome-based mitochondrial delivery of circRNA mSCAR alleviates sepsis by orchestrating macrophage activation. *Adv Sci (Weinh)* 2023;**10**:e2205692.
158. Kimiz-Gebologlu I, Oncel SS. Exosomes: large-scale production, isolation, drug loading efficiency, and biodistribution and uptake. *J Control Release* 2022;**347**:533−43.
159. Erana-Perez Z, Igartua M, Santos-Vizcaino E, Hernandez RM. Genetically engineered loaded extracellular vesicles for drug delivery. *Trends Pharmacol Sci* 2024;**45**:350−65.
160. Mohsen MO, Zha L, Cabral-Miranda G, Bachmann MF. Major findings and recent advances in virus-like particle (VLP)-based vaccines. *Semin Immunol* 2017;**34**:123−32.
161. Zhang P, Narayanan E, Liu Q, Tsybovsky Y, Boswell K, Ding S, et al. A multiclade env-gag VLP mRNA vaccine elicits tier-2 HIV-1-neutralizing antibodies and reduces the risk of heterologous SHIV infection in macaques. *Nat Med* 2021;**27**:2234−45.
162. Mejia-Mendez JL, Vazquez-Duhalt R, Hernandez LR, Sanchez-Arreola E, Bach H. Virus-like particles: fundamentals and biomedical applications. *Int J Mol Sci* 2022;**23**:8579.

163. Nooraei S, Bahrulolum H, Hoseini ZS, Katalani C, Hajizade A, Easton AJ, et al. Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers. *J Nanobiotechnol* 2021;**19**:59.
164. Banskota S, Raguram A, Suh S, Du SW, Davis JR, Choi EH, et al. Engineered virus-like particles for efficient *in vivo* delivery of therapeutic proteins. *Cell* 2022;**185**:250-65.e16.
165. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov* 2019;**18**: 358−78.
166. Meganck RM, Borchardt EK, Castellanos Rivera RM, Scalabrino ML, Wilusz JE, Marzluff WF, et al. Tissue-dependent expression and translation of circular RNAs with recombinant AAV vectors *in vivo*. *Mol Ther Nucleic Acids* 2018;**13**:89−98.
167. Mecozzi N, Nenci A, Vera O, Bok I, Falzone A, DeNicola GM, et al. Genetic tools for the stable overexpression of circular RNAs. *RNA Biol* 2022;**19**:353−63.
168. McKinlay CJ, Vargas JR, Blake TR, Hardy JW, Kanada M, Contag CH, et al. Charge-altering releasable transporters (CARTs) for the delivery and release of mRNA in living animals. *Proc Natl Acad Sci U S A* 2017;**114**. E448-e56.
169. Haabeth OAW, Lohmeyer JJK, Sallets A, Blake TR, Sagiv-Barfi I, Czerwinski DK, et al. An mRNA SARS-CoV-2 vaccine employing charge-altering releasable transporters with a tlr-9 agonist induces neutralizing antibodies and T cell memory. *ACS Cent Sci* 2021;**7**: 1191−204.
170. Li Z, Amaya L, Ee A, Wang SK, Ranjan A, Waymouth RM, et al. Organ- and cell-selective delivery of mRNA *in vivo* using guanidinylated serinol charge-altering releasable transporters. *J Am Chem Soc* 2024;**146**:14785−98.
171. Zhu D, Li J, Wang L, Li Q, Wang L, Song B, et al. Hydrophobic collapse-driven nanoparticle coating with poly-adenine adhesives. *Chem Commun (Camb)* 2021;**57**:3801−4.
172. ClinicalTrials.gov. *Study of TI-0093 injection with recurrent/metastatic HPV-16 positive solid tumors*. ClinicalTrialsgov; 2025. Available from: https://clinicaltrials.gov/study/NCT07081984.
173. Feng Z, Zhang X, Zhou J, Li Q, Chu L, Di G, et al. An *in vitro*-transcribed circular RNA targets the mitochondrial inner membrane cardiolipin to ablate EIF4G2(+)/PTBP1(+) pan-adenocarcinoma. *Nat Cancer* 2024;**5**:30−46.
174. Zhang C, Hu J, Liu Z, Deng H, Xiao J, Yi Z, et al. Hsa_circ_0000520 suppresses vasculogenic mimicry formation and metastasis in bladder cancer through Lin28a/PTEN/PI3K signaling. *Cell Mol Biol Lett* 2024;**29**:118.
175. Imani S, Li X, Chen K, Maghsoudloo M, Jabbarzadeh Kaboli P, Hashemi M, et al. Computational biology and artificial intelligence in mRNA vaccine design for cancer immunotherapy. *Front Cell Infect Microbiol* 2024;**14**:1501010.
176. Long X, Sun K, Lai S, Liu Y, Su J, Chen W, et al. Artificial intelligence and anti-cancer drugs' response. *Acta Pharm Sin B* 2025;**15**: 3355−71.
177. Chen Y, Wang J, Wang C, Liu M, Zou Q. Deep learning models for disease-associated circRNA prediction: a review. *Briefings Bioinf* 2022;**23**:bbac364.
178. Tian Y, Zou Q, Wang C, Jia C. MAMLCDA: a meta-learning model for predicting circRNA-disease association based on MAML combined with CNN. *IEEE J Biomed Health Inform* 2024;**28**: 4325−35.
179. Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, et al. Immune epitope database analysis resource. *Nucleic Acids Res* 2012;**40**:W525−30.
180. Zhang H, Zhang L, Lin A, Xu C, Li Z, Liu K, et al. Algorithm for optimized mRNA design improves stability and immunogenicity. *Nature* 2023;**621**:396−403.
181. Sample PJ, Wang B, Reid DW, Presnyak V, McFadyen IJ, Morris DR, et al. Human 5′ UTR design and variant effect prediction from a massively parallel translation assay. *Nat Biotechnol* 2019;**37**:803−9.
182. Fu H, Liang Y, Zhong X, Pan Z, Huang L, Zhang H, et al. Codon optimization with deep learning to enhance protein expression. *Sci Rep* 2020;**10**:17617.
183. Porollo AA, Adamczak R, Meller J. POLYVIEW: a flexible visualization tool for structural and functional annotations of proteins. *Bioinformatics* 2004;**20**:2460−2.
184. Zhang L, Xiao R, Gao W, Garcia J, Pan X, Daristotle JL, et al. Polyanhydride-based microparticles for programmable pulsatile release of diphtheria toxoid (DT) for single-injection self-boosting vaccines. *Adv Mater* 2025;**37**:e2501168.
185. Ngo LH, Bert AG, Dredge BK, Williams T, Murphy V, Li W, et al. Nuclear export of circular RNA. *Nature* 2024;**627**:212−20.
186. Wang X, Xu W, Li J, Shi C, Guo Y, Shan J, et al. Nano-omics: frontier fields of fusion of nanotechnology. *Smart Med* 2023;**2**: e20230039.
187. Conn SJ, Pillman KA, Toubia J, Conn VM, Salmanidis M, Phillips CA, et al. The RNA binding protein quaking regulates formation of circRNAs. *Cell* 2015;**160**:1125−34.
188. Chen R, Wang SK, Belk JA, Amaya L, Li Z, Cardenas A, et al. Engineering circular RNA for enhanced protein production. *Nat Biotechnol* 2023;**41**:262−72.
189. Park OH, Ha H, Lee Y, Boo SH, Kwon DH, Song HK, et al. Endoribonucleolytic cleavage of m(6)A-containing RNAs by RNase P/MRP complex. *Mol Cell* 2019;**74**:494−507 e8.
190. Chen YG, Kim MV, Chen X, Batista PJ, Aoyama S, Wilusz JE, et al. Sensing self and foreign circular RNAs by intron identity. *Mol Cell* 2017;**67**:228-38.e5.
191. Amaya L, Grigoryan L, Li Z, Lee A, Wender PA, Pulendran B, et al. Circular RNA vaccine induces potent T cell responses. *Proc Natl Acad Sci U S A* 2023;**120**:e2302191120.
192. Kang M, Jordan V, Blenkiron C, Chamley LW. Biodistribution of extracellular vesicles following administration into animals: a systematic review. *J Extracell Vesicles* 2021;**10**:e12085.
193. Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK. Virus-like particle vaccines: immunology and formulation for clinical translation. *Expert Rev Vaccines* 2018;**17**:833−49.
194. Bakos T, Meszaros T, Kozma GT, Berenyi P, Facsko R, Farkas H, et al. mRNA-LNP COVID-19 vaccine lipids induce complement activation and production of proinflammatory cytokines: mechanisms, effects of complement inhibitors, and relevance to adverse reactions. *Int J Mol Sci* 2024;**25**:3595.
195. Segel M, Lash B, Song J, Ladha A, Liu CC, Jin X, et al. Mammalian retrovirus-like protein PEG10 packages its own mRNA and can be pseudotyped for mRNA delivery. *Science* 2021;**373**:882−9.