REVIEW

CircRNA-based therapeutics: Current opinions and clinical potential

Hanyuan Liu,[1,2,4] Xuelin Yao,[3,4] Ying Zhou,[2,*] and Liang Chen[1,*]

*Correspondence: anqingcl@ustc.edu.cn (L.C.); caddiezy@ustc.edu.cn (Y.Z.)

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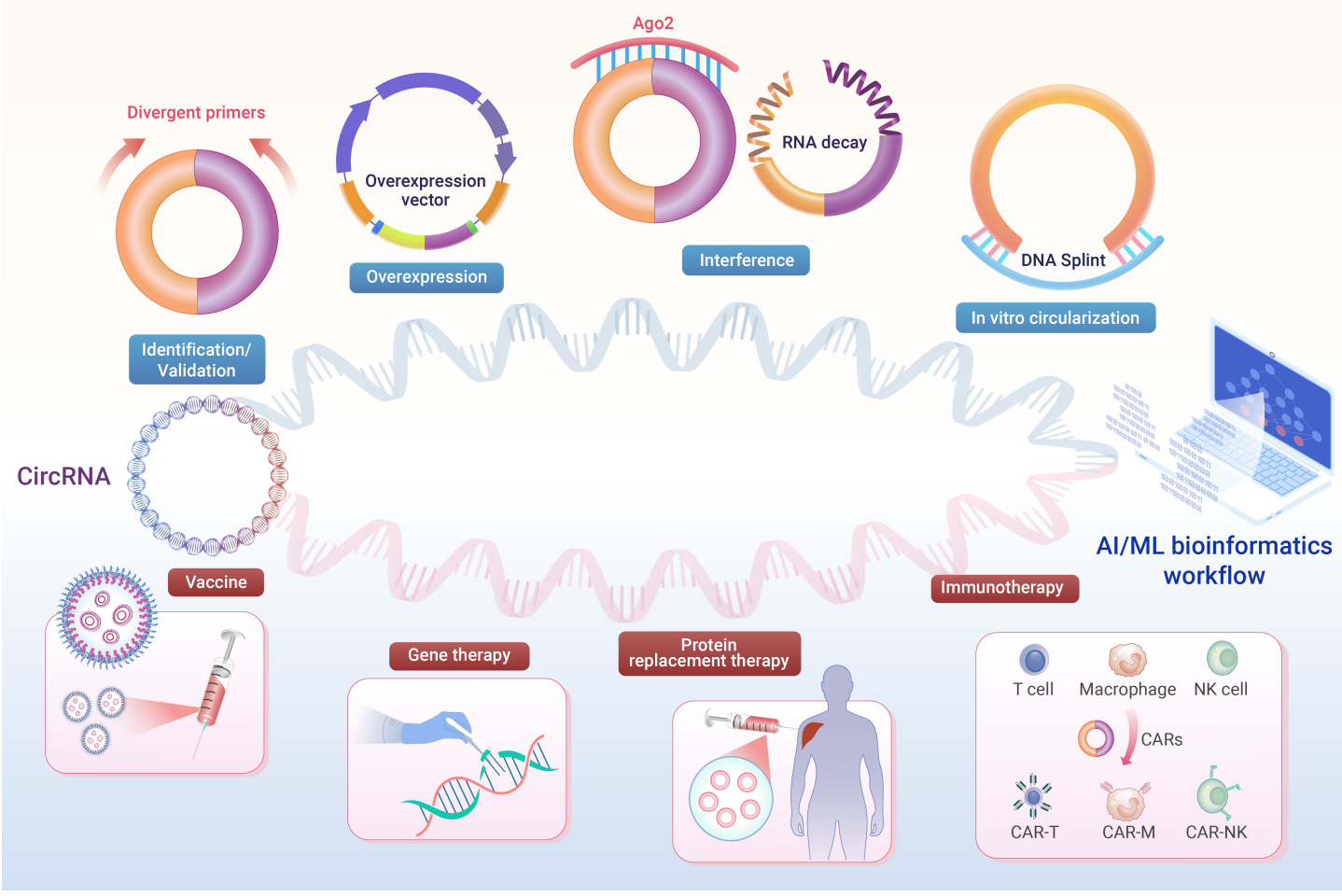
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Circular RNAs (circRNAs) are critical regulators implicated in multiple pathological conditions.
- CircRNAs manipulation and delivery options provide circRNAs research and applications opportunities.
- CircRNA-based therapeutic strategies have been emerging.

REVIEW

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Hanyuan Liu,[1,2,4] Xuelin Yao,[3,4] Ying Zhou,[2,*] and Liang Chen[1,*]

[1]Department of Cardiology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, China
[2]Department of Obstetrics and Gynecology, Core Facility Center, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, China
[3]Department of Endocrinology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, China
[4]These authors contributed equally
*Correspondence: anqingcl@ustc.edu.cn (L.C.); caddiezy@ustc.edu.cn (Y.Z.)

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Circular RNAs (circRNAs) are single-stranded, covalently closed RNA molecules that perform diverse roles in various cellular processes and have been implicated in many pathological conditions. Owing to their intrinsic stability and low immunogenicity, circRNAs have garnered significant interest for their therapeutic potential in multiple diseases, with advancements in efficient in vitro production methods and optimized delivery systems. In this review, we provide a comprehensive overview of current knowledge on circRNA biogenesis and functions, and summarize recent advances in various technologies for circRNA research, including their profiling, validation, and biosynthesis. We also discuss key delivery strategies and therapeutic applications, highlighting the promising prospects and current challenges for the clinical development of circRNA-based therapeutics. Research to date has shown that circRNAs are not merely splicing errors and that circRNA-based therapeutic platforms may have superior application prospects from bench to bedside.

INTRODUCTION

Since the discovery of circular RNAs (circRNAs) in the 1970s,[1,2] the field has evolved significantly, culminating in the recent development of circRNA vaccines in 2022.[3] In 1986, Kos et al. confirmed the existence of the human hepatitis δ virus circRNA genome by electron microscopy.[4] During the 1990s, researchers identified several transcripts that were abnormally spliced, with scrambled exons joining at the consensus splice sites of endogenous circRNA.[5-7] Further studies demonstrated that long inverted repeats flanking the mouse *Sry* gene are essential for the circularization of the circular Sry transcript,[8,9] and that engineered circRNA can be translated both *in vitro*[10] and *in vivo*.[11]

For decades, circRNAs were dismissed as splicing errors or by-products of pre-mRNA splicing.[7] However, the early 21st century saw a surge of interest in circRNAs, leading to exponential growth in the field.[12] CircRNAs have emerged as one of the primary transcript isoforms for hundreds of human genes.[13] The emergence of high-throughput RNA sequencing (RNA-seq) technologies, along with bioinformatics algorithms and experimental techniques for circRNA profiling, has revealed the broad expression of circRNAs and their biological functions across various cell types and tissues.[14-21]

CircRNAs are generally more stable than linear transcripts[22] and exhibit cell type, tissue, and developmental stage specificity.[23] Many dysregulated circRNAs have been identified as reliable biomarkers for diagnosing and predicting the progression of various diseases.[24,25] With a better understanding of the high stability and low immunogenicity of natural circRNAs, there is a growing interest in the development of circRNA synthesis techniques, and the applications of engineered circRNAs for the treatment of diseases have been explored (Figure 1).[26-30] This review comprehensively summarizes current knowledge of circRNAs and the latest techniques for their detection and manipulation. Our discussion highlights the potential of circRNAs for clinical treatment, and outlines future perspectives and challenges in the field of circRNA-based therapeutics.

OVERVIEW OF CIRCRNAS

Biogenesis and properties of circRNAs

CircRNAs are generated by a back-splicing event from precursor RNA (pre-RNA), resulting in four subclasses of circRNAs based on their distribution and composition (Figure 2): exonic circRNAs (EcircRNAs),[31] intronic circRNAs (ciRNAs),[32] exon-intron circRNAs (EIcircRNAs), and mitochondria encoded circRNAs (mecciRNAs).[33] Generating circRNA requires that the donor splice site of the exon is not linked to the acceptor splice site of the downstream exon in linear splicing, but instead to the upstream acceptor site.[34-36] The exact mechanism by which the spliceosome selects specific exons for circularization remains unclear, but it involves bringing the two introns flanking the back-splicing exons into close proximity.[37] Three mechanisms are involved in this step: intron-pairing driven circularization,[38] RNA-binding protein (RBP)-driven circularization,[39] and intronic lariat precursors that escape debranching give rise to ciRNAs.[40] Overall, a combination of both intron-pairing and RBP factors are likely to provide a more complex set of processes that affect circRNA biogenesis.

Nuclear transport and subcellular localization of circRNAs

Most EcircRNAs are cytoplasmic, whereas EIciRNAs and ciRNAs are predominantly nuclear.[41] The transport of circRNAs includes length-dependent nuclear export, m6A-mediated nuclear export, and intercellular transport of circRNAs via extracellular exosomes.[35,42] Huang et al. found that circRNA localization is actively controlled by Drosophila Hel25E and its human homolog UAP56 in a length-dependent manner.[43] In addition, our lab identified the conserved Exportin 4 (XPO4) as an essential regulator in modulating the nuclear export of a subset of ecircRNAs.[44] XPO4 deficiency triggers the pervasive formation of circRNA: genomic DNA hybrids (circRNA:DNA R-loops, ciR-loops) and DNA damage in the nucleus, leading to infertility and neurological defects.[44,45] It is known that imbalanced subcellular localization of circRNAs and defects in nuclear export leads to cancer transformation and disrupted intracellular signaling.[46,47] Therefore, further investigation of the nuclear export mechanisms of circRNAs is valuable for uncovering their biological functions and physiological relevance.

Initially, nuclear-cytoplasmic fractionation followed by real-time quantitative polymerase chain reaction (RT-qPCR) can indicate the cellular localization of circRNA. RNA-FISH can use DNA probes targeting back-splicing junction sites to quantify circRNAs and confirm their localization in cells.[48,49] Ribosome profiling (RIBO-seq) uses deep sequencing of ribosome-protected mRNA fragments to monitor translation with speed, accuracy, and scale that compares favorably to methods used to track mRNA levels.[50] As RIBO-seq advances, more circRNAs are being found to be translated in diverse organisms, and a subset of them can produce functional polypeptides.[51]

In addition, cytoplasmic and nuclear circRNAs can be biochemically isolated in different proportions and analyzed for different circRNA species on a genome-wide level using high-throughput approaches. For example, CeFra-seq has been used to detect RNA localization in multiple cellular fractions,[52] while APEX-seq investigates comprehensive localization patterns for different categories of circRNA in multiple subcellular locations.[53] Several databases, including CircVIS,[54] MNDR v3.0,[55] and RNALocate,[56] have been established to provide a genome-wide summary of the subcellular localization of circRNAs. Moreover, artificial intelligence (AI) techniques such as CircLocNet,[57] mRNALoc,[58] and RNAlight[59] have been developed to predict the subcellular localization of circRNAs.

Biological functions of circRNAs

Nuclear circRNAs modulate gene expression by regulating transcription or alternative splicing patterns, while cytoplasmic circRNAs function in various ways, such as acting as microRNA (miRNA) sponges[21] and translation

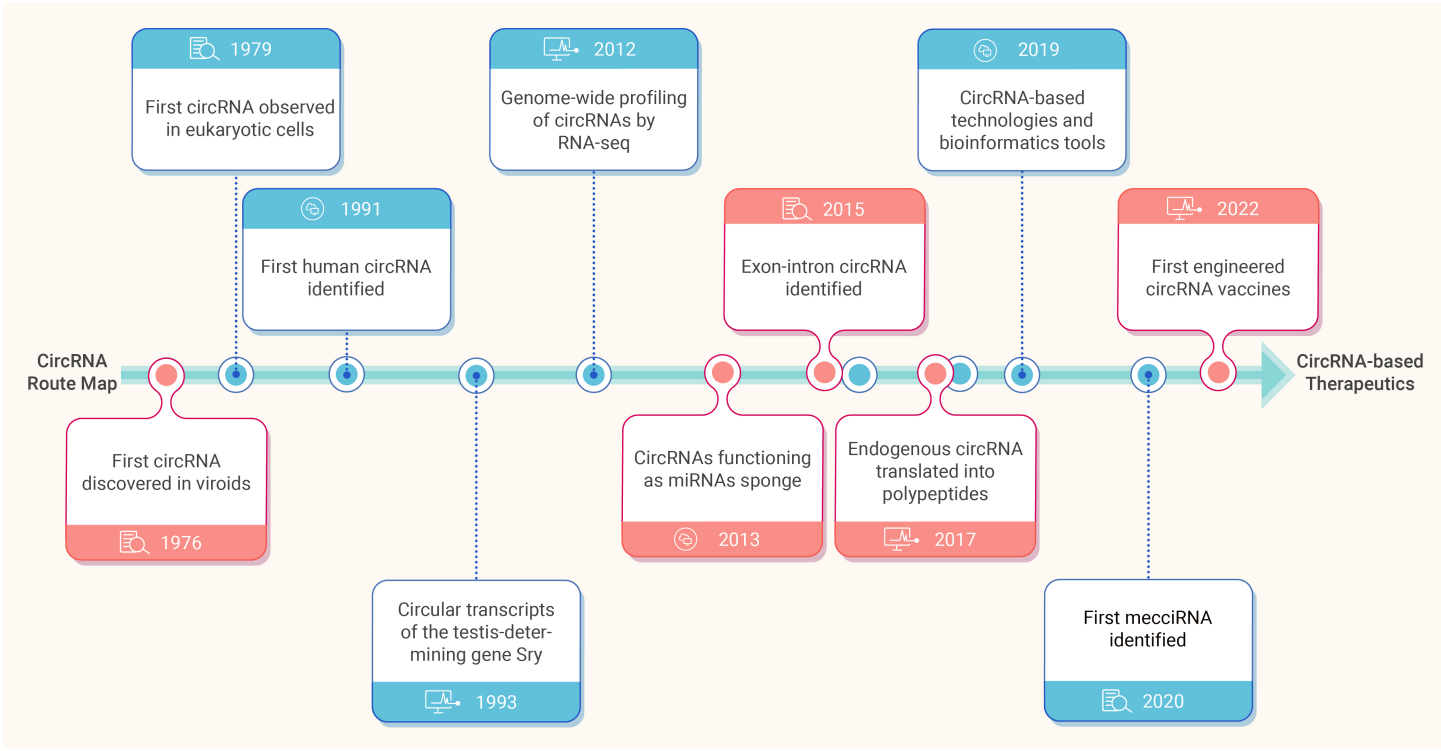

Figure 1. Route map of circRNAs. The development of circRNA discoveries is illustrated In 1976, circRNA was identified in plant viroids by electron microscopy.[1] Similar methods were used to detect circRNAs in the cytoplasm of eukaryotic cells in 1979.[2] The first human circRNA was identified in 1991.[5] In 1993, the mouse testis-specific circRNA was shown to be an abundant transcript in the testis.[9] In 2012, genome-wide profiling of circRNAs was demonstrated by RNA-seq.[13] In 2013, circRNA was shown to function as miRNA sponge.[20,21] In 2015, exon-intron circRNAs was identified in the nucleus of human cells.[33] In 2017, circRNAs were shown to be translated into polypeptides.[19] In 2019, circRNA-based technologies and bioinformatics tools were developed for detecting circRNA, such as CircFunBase.[16] In 2020, hundreds of circular RNAs (mecciRNAs) encoded by the mitochondrial genome were first identified.[29] In 2022, engineered circRNA vaccines are being used for the first time in the treatment and prevention of COVID-19 and melanoma.[3,30]

templates.[31] CircRNAs also interact with proteins through specific binding sites in RBPs.[49] While proteins contain only a limited number of circRNA binding sites, the exceptional tertiary structure of circRNAs provides them with significant affinity and flexibility for RBP binding.[60] In addition, some circRNAs can bind to mRNAs to regulate their stability and translation, which in turn affects gene expression in various physiological processes.[61-62] In exceptional cases, some circRNAs have been identified as transcripts that can be translated into peptides in a cap-independent manner.[63] The growing understanding of the complex functions of circRNA underscores the need for further research and application in disease treatment.

METHODS FOR CIRCRNA STUDIES

Recent advancements in the identification and validation of circRNAs have significantly facilitated the initial steps toward RNA-based therapeutics.

Identification of circRNAs

Early studies relied on cDNA amplification using PCR with divergent primers. The advent of deep sequencing techniques, coupled with computational methods to analyze transcriptome datasets, has enabled the discovery of circRNAs on a genome-wide scale. Two commonly used protocols for circRNA library construction include obtaining ribosomal RNA-depleted RNA ($rRNA^-$ library),[64] and generating libraries by depleting both rRNA and poly(A)$^+$ RNA (rRNA- poly(A)$^-$ library) or by using exonuclease (RNase R) treatment to digest most linear RNAs ($rRNA^-$ RNase R^+ library).[38] However, linear non-polyadenylated RNAs resistant to RNase R with highly structured ends can interfere with downstream analysis. To address this issue, Xiao et al.[65] and Pandey et al.[66] used advanced methods to identify highly enriched circRNA by efficiently removing linear RNAs containing G-quadruplexes or structured 3′ ends.

Genome-wide detection and enrichment techniques have been employed to analyze non-polyadenylated transcriptomes and RNase R-treated transcriptomes, including non-ribosomal RNA sequencing (ribo- RNA-seq),[20] non-ribosomal and non-polyadenylated RNA sequencing (ribo- poly(A)- RNA-seq),[13,38] and RNase R-treated RNA-seq.[67] CircRNAs are often measured and distinguished using techniques such as enhanced RNase R degradation of linear RNAs,[65] normalization of RNA-seq fragments mapped to circRNA back-splicing junction sites,[68] and the CIRC score to assess expression of circular and related linear RNAs.[69] For single-molecule real-time sequencing, PacBio, and Oxford Nanopore have been developed, both of which are capable of obtaining full-length transcripts and quantifying them without bias.[70,71] To date, several strategies for sequencing full-length circRNA isoforms based on nanopore sequencing technology have been developed to annotate circRNAs. IsoCirc generated a comprehensive catalog of 107,147 full-length circRNA isoforms from 12 human tissues and HEK293 cells.[72] CIRI-long[73] and circFL-seq[74] provided conclusive evidence of circRNA discovery using rolling circle amplification (RCA) and nanopore long-read sequencing. Our group recently developed a framework, FEICP, which is capable of efficiently detecting EIciRNAs from high-throughput sequencing (HTS) data.[75]

Different computational pipelines use various approaches to identify circRNAs. FindCirc uses anchor sequences to identify back-splicing sites and has been used to identify circRNAs in humans, mice, and *C. elegans*.[20] Another pipeline Segemehl identifies back-splicing junctions using a *de novo*-based approach,[76] while the third pipeline CIRI identifies circRNAs from transcriptome data using multiple filtering strategies and has identified, and validated the prevalence of intronic/intergenic circRNAs with their specific fragments in the human transcriptome.[77] Although circRNA-seq has provided new insights into the RNA landscape, sequencing fragmentation has led to significant noise and loss of important information, such as the intricate alternative splicing of circRNAs. CIRCexplorer and CIRCexplorer2 pipeline have been reported as circRNA prediction tools with reliable circRNA prediction outputs, the improved CIRCexplorer2 pipeline has been further developed to annotate the intricate alternative back-splicing and alternative splicing events of circRNAs.[68]

Quantification and detection of circRNAs

Traditional techniques for circRNA profiling, such as Northern blotting,[78] RT-

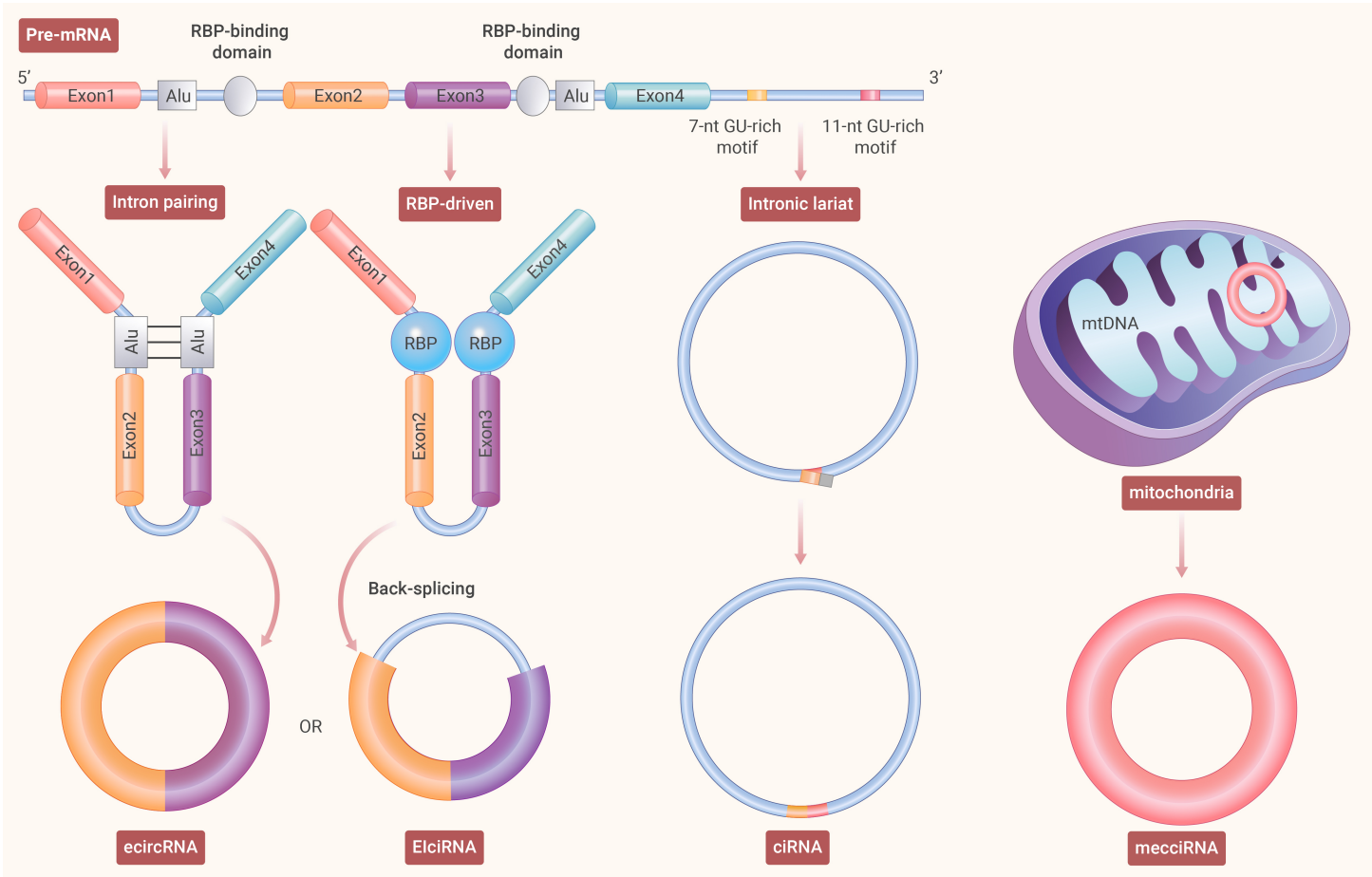

Figure 2. The classification and biogenesis of circRNAs The flanking introns of circularized exons generate exonic circRNA (ecircRNA) or exon-intron circRNA (eIciRNA) by direct insertion or binding to RBP binding sites. Intronic circRNA (ciRNA) is derived from an intron by prevention of intron debranching after splicing. Mitochondrial circRNAs (mecciRNAs) are circularized by a splicing-independent mechanism.

qPCR,[20] and microarray analysis,[79] are effective but have limitations. Innovative assays, including reverse transcription droplet digital PCR,[80] loop-mediated isothermal exponential amplification,[81] and RCA,[82] and nanostring technologies nCounter assays[80] have been developed to overcome limitations and improve sensitivity and specificity in circRNA detection. These novel methods hold promise for accurate profiling of circRNAs and may lead to their broader clinical application.[83]

Effective isolation of circRNAs is essential for their detection and further analysis. Various biochemical methods have been proposed to increase the yield of circRNA in samples, such as two-dimensional denaturing polyacrylamide gel electrophoresis and rRNA or poly (A) depletion.[84] However, these techniques are expensive and time-consuming. Therefore, it is imperative to develop an efficient and affordable pretreatment assay for circRNA isolation. The development of a robust tool is crucial for the analysis of circRNA in large-scale single-cell gene expression measurements. Elucidating the biology of circRNA requires technologies capable of detecting the temporal and spatial expression sequence of specific circRNA patterns in tissues.[85] However, due to the low levels of circRNA in cells,[86] it is essential to develop a more efficient and sensitive method of circRNA detection for *in vivo* applications. Accurate and efficient enrichment of circRNAs facilitates the comprehensive study of a large number of circRNAs. The identification of circRNAs and their associated proteins and RNA molecules requires the application of RNA pull-down and RNA immunoprecipitation-based assays, which allow the identification of pulled-down proteins via end-labeled biotinylated RNA oligonucleotides and immunoblotting.[87]

CircRNA-based deep learning algorithms

Advances in digital technology and sophisticated computational algorithms have stimulated interest in applying AI, especially deep learning (DL)-based AI, to circRNA research.[88] DL has provided deep insights into disease pathogenesis, discovery of potential diagnostic and therapeutic targets, and prediction of circRNA-disease association. DL-based computational models consist of the following three steps. First, circRNA-disease similarity or semantic information and circRNA-disease association information are integrated to build a heterogeneous similarity network. Second, models extract latent features based on the initial network. Finally, a machine learning classifier is trained to predict the association between disease and circRNA. It is crucial to select an appropriate classification or regression algorithm according to the structure of an overall prediction model, as different prediction algorithms have different strengths. As shown in Table S1, several circRNA-disease association prediction methods based on DL are presented.[89-135] DeepDCR is designed to predict potential disease-related circRNAs and constructed a heterogeneous biological network including 17,961 circRNAs, 469 miRNAs, and 248 diseases.[134] CIRI-deep provides a back-splicing junction read-independent approach to predict circRNA regulation between biological samples using total or poly(A) RNA-seq data.[135]

DL has demonstrated its great advantages and uniqueness in various tasks as a competitive approach to traditional machine learning methods.[136] Compared with other existing methods, including VGAELDA, SIMCCDA, NCPLDA, GMNN2CD, MLMKDNN, and GCNMDA, CLCDA achieves the best performance in predicting potential disease-related circRNAs and has strong generalization.[136] All of the above DL methods may provide more reliable candidate circRNAs for subsequent biological experiments. However, these methods also have some limitations. Some DL-based prediction methods, such as similarity calculation, rely heavily on known circRNA-disease associations, which cannot be applied to new diseases without known associations with circRNAs.[127] The circRNA-disease association databases have only been established in recent years, and similar circRNAs are associated with different diseases in the databases. For example, the imbalance of data in

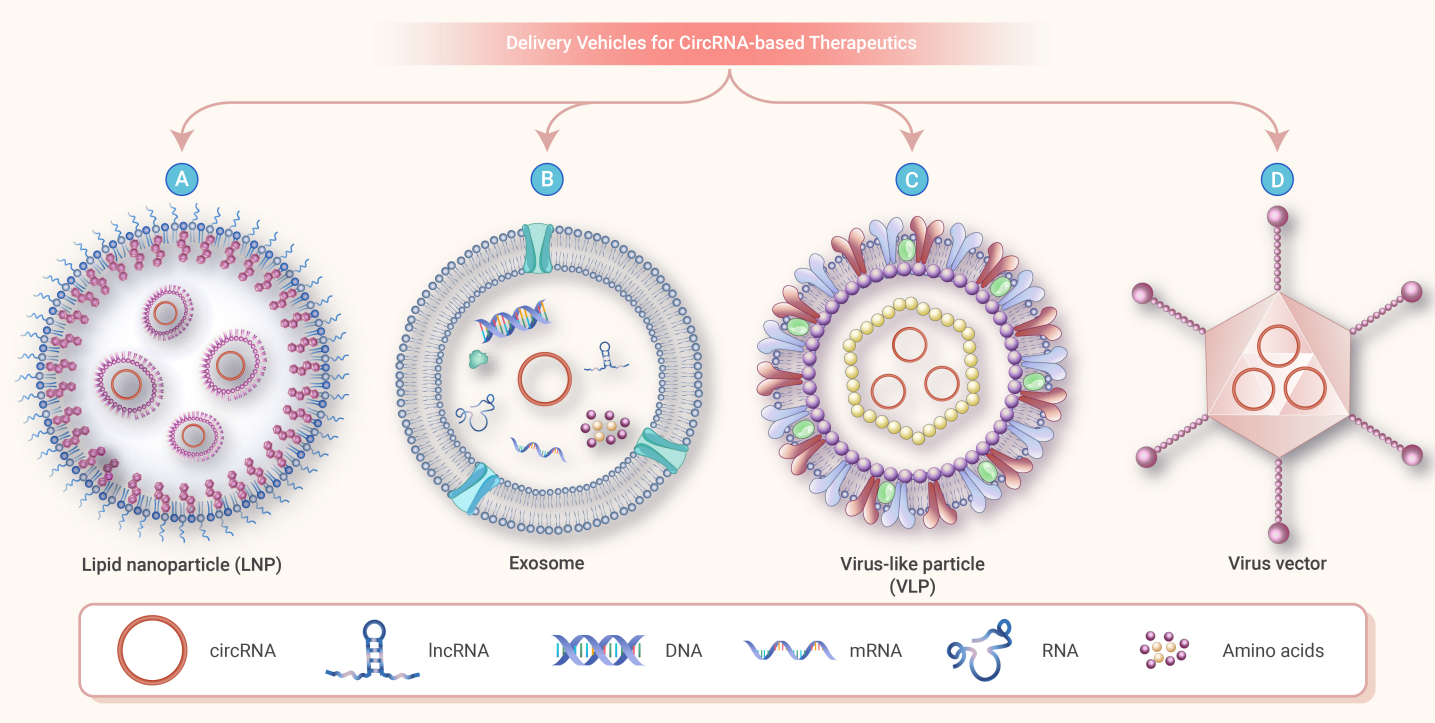

Figure 3. Various delivery vehicles for circRNA-based therapeutics General components and structure of a lipid nanoparticle (LNP), exosome, virus-like particle (VLP), and viral vector.

CircR2Disease leads to a similar top 10 associated circRNA of different diseases. The incomplete database may affect the prediction performance of circRNA-disease association prediction. Existing databases lack verified, reliable negative samples, while the performance of supervised DL depends on the sample quality. Although the reasonable fusion of multiview similarity into synthetic similarity networks improves the quality of synthetic similarity networks, these similarity fusion methods are challenging due to different computational rules and suffer from different levels of inaccuracy in these multiview data sources.[89,127] It is very difficult to obtain appropriate feature representations for circRNAs and diseases that include all the critical information from the similarity networks and the association matrix. This is also a challenge for all DL-based methods for predicting circRNA-disease associations.

MANIPULATION OF CIRCRNA EXPRESSION LEVELS

Knockdown of circRNAs by RNA interference

RNA interference (RNAi) has been pivotal in gene silencing for clinical applications.[137] Among these, small interfering RNA (siRNA) and short hairpin RNA (shRNA) are the most valuable applications.[138] SiRNAs undergo cytoplasmic processing by Dicer and are subsequently incorporated into the RNA-induced silencing complex (RISC).[139] SiRNAs provide researchers with a versatile means to modulate target gene expression.[140] To silence intracellular circRNA expression in mammals, shRNAs can be cloned into exogenous expression vectors.[141] Plasmid and viral vector systems can effectively transcribe shRNA precursors, which can lead to significant circRNA knockdown via the RNAi pathway.[142] In addition to siRNAs and shRNAs, antisense oligonucleotides (ASOs) bind to circRNA targets via the Watson-Crick base pairing mechanism and promote their degradation via endonuclease activity.[143] To knock down circRNAs without affecting the corresponding linear mRNA, the backsplice junction unique to circRNAs is usually targeted. Modified ASOs complementary to backsplice junction sequences can be used to selectively modulate robust knockdown of endogenous circRNAs *in vitro*. This tool provides a useful means to explore the biological roles of circRNAs in loss-of-function studies in cultured cells and animal models.[144]

Overexpression of circRNAs

Although mammalian expression plasmids can achieve transient circRNA overexpression in cultured cells, most cell biology research requires long-term ectopic expression.[145] Most studies rely on circularization caused by intron pairing to overexpress circRNAs, and a plethora of expression vectors have been created to enhance their expression for gain-of-function studies.[26] These vectors typically contain split reporter exons flanked by two introns with complementary regions that facilitate backsplicing of the pre-mRNA.[146] Adenoviral and lentiviral vectors carrying intron-containing cassettes have been widely used to promote backsplicing in circRNA transfer applications *in vivo*.[147] Standard gain-of-function approaches have significant limitations in the objective study of circRNAs. Efficient transposon-mediated systems are being used for long-term overexpression of circRNA constructs in cells and a mouse model of hepatocellular carcinoma.[145] The use of these tools and techniques allows for more comprehensive analyses of circRNA functionality *in vitro* and *in vivo*.

Gene editing-mediated circRNA knockout or knockdown

Most circRNAs contain overlapping sequences with their cognate linear RNAs from the same gene loci, making them difficult to distinguish. However, several gene editing techniques, including the CRISPR-Cas9 system, base editing, prime editing, and adenosine deaminase-acting on RNA (ADAR) RNA editing, have been utilized for circRNA knockout or knockdown with greater specificity than RNA interference (RNAi).[148,149]

The CRISPR/Cas9 platform uses a single endonuclease and a single guide RNA (gRNA) to induce precise, sequence-specific DNA double-strand breaks (DSBs) in the genome.[150] CRISPR/Cas9 knocks out circRNAs by disrupting the pairing of introns flanking circularized exons that occur during circRNA biogenesis. CRISPR/Cas9 has been used to knock out circGCN1L1 and circHIPK3 *in vitro* without affecting their linear mRNA.[151,152] It is more challenging to use the CRISPR/Cas9 system to target intronic sequences involved in circRNA production compared to RNAi-based strategies that directly target the back splice junction. RNA-targeting type VI CRISPR effectors, known as Cas13a, Cas13b and Cas13d RNases, can be directed to cleave single-stranded RNA targets in mammalian cells.[153] Instead of using CRISPR/Cas9 to knock out circRNAs by targeting the intronic complementary sequences flanking circularized exons or gene loci, CRISPR/Cas13 can directly target the reverse splice junction of circRNAs. Several studies have evaluated the use of a CRISPR/Cas13d system for circRNA knockdown *in vitro*. Cas13d and

shRNA achieved similar circRNA knockdown effects, while Cas13d produced significantly fewer off-target effects.[154] In addition, a functional screen of CRISPR/Cas13d and shRNA-based circRNAs was performed and it was found that shRNA screens resulted in significantly higher false positive rates.[154] RfxCas13d has been used against many other circRNAs and showed specific and robust knockdown of all circRNAs.[155]

Base editing has recently been developed to directly alter targeted base pairs.[156] This technology alters nucleotide changes without generating DSBs, homology-directed repair (HDR), or donor DNA templates by fusing a base conversion enzyme to deactivated Cas9.[148,156] Some circRNAs can be specifically depleted using base editing systems to circularize their predominantly backsplice sites and efficiently repress the production of circular but not linear RNAs.[157,158] CDR1as/ciRS-7 and circZNF292 were specifically depleted with no apparent effect on the expression of their cognate linear RNAs when base editors targeted sites predominantly involved in back-splicing, providing an efficient and specific method for endogenous circRNA knockout using base editors *in vivo*.[158] Prime editors consist of a Cas9 nickase linked to reverse transcriptase and a prime editing guide RNA.[159] Unlike base editing, prime editing mediates directed insertions, deletions, and all 12 possible base-to-base conversions without introducing DSBs or donor templates, greatly expanding the capabilities and applications of genome editing.[160] RNA editing makes single nucleotide changes by fusing with endogenous ADAR enzymes.[161] However, this technology is still immature, and ADAR-recruiting RNA of a certain length may cause off-target editing of adjacent bases.[162]

Synthetic circRNA techniques

The widespread presence of circRNAs *in vivo* and the investigation of their structural and functional properties have created a need for effective methods to produce circular RNAs *in vitro*.[27] Circularization of linear RNAs is a critical process in the synthesis of circular RNA *in vitro*. Three methods are used for circularization by joining the two ends of linear RNAs. The first involves the addition of condensation reagents that form a phosphodiester bond between the 5′-phosphate and 3′-hydroxyl groups of the linear RNA.[163] However, this method inevitably produces by-products such as 2′, 5′-phosphodiester linkages from the same condensation reaction. Next, circularization of linear RNAs can be achieved by enzymatic ligation with DNA/RNA ligases.[164,165] A caveat of this approach, however, is the likelihood of concatemer formation, which can reduce the overall yield of circular RNA. Finally, ribozymes can be used to conveniently facilitate the production of circular RNAs. One method involves engineered hairpin ribozyme variants that fold into two alternative conformations by truncating their 5′- and 3′-terminus, thereby producing a 5′-OH group and a 3′-terminal 2′,3′-cyclic phosphate.[166] Another ribozyme-assisted method of circRNA production relies on a spontaneous group I intron self-splicing system.[167] Chemical and enzymatic protocols are available in most cases, but most are better suited for small and medium-sized RNAs.[168] Many natural ribozymes have rarely been used for engineering purposes to create tools to facilitate RNA ligation/circularization on a preparative scale. *In vitro* transcription often leads to terminal heterogeneity, which significantly reduces the yield of circularization for ligation. Although this challenge can be overcome by using specialized protocols,[169] improving the efficiency of the circularization of longer RNA remains a daunting task.

In addition to enhancing circRNA expression using plasmids, synthetic circRNAs hold promise for providing superior stability and sustained gene expression in cells.[170] *In vitro* circularization of RNA can be used to produce highly purified circRNA molecules and to engineer efficient miRNA sponges.[171] Further investigation of circRNA circularization may be useful in other transcriptome and genome engineering modalities, such as RNAi, ASOs, and guide RNAs in CRISPR-Cas.[172] It is worth noting that artificial circRNA can not only be translated into proteins for disease prevention and treatment, but also exert potential effects through non-coding functions.[171] Various artificial circRNAs have been developed to act as endogenous miRNA sponges.[173] In addition, engineered circular RNA aptamers can serve as valuable tools to modulate various intracellular pathways by acting as efficient metabolite biosensors and fluorescent sensors.[47]

CURRENT DELIVERY SYSTEMS FOR CIRCRNAS

Studies have shown that mRNA and siRNA can be delivered safely and efficiently to target human tissues and for successful transfection.[174] However, the delivery of circRNA-based therapeutics to cells presents significant challenges. First, nucleic acids can interact with serum proteins, be taken up by phagocytes, and be degraded by endogenous nucleases. Second, nucleic acids are unable to penetrate the anionic lipid bilayer of cell membranes due to their negative charge and hydrophilicity. Thus, the delivery system is critical to protect exogenous circRNA-based therapeutics and facilitate their transport into cells. This section will introduce several innovative delivery systems and discuss the delivery strategies of circRNA based on relevant studies (Figure 3).

Lipid nanoparticles

Lipid nanoparticles (LNPs) are defined as submicron particles containing ionizable cationic lipids in addition to other types of lipids and encapsulated nucleic acid cargo.[175] LNPs can act as a protective capsule for nucleic acids such as siRNA, mRNA, and circRNA, preventing enzymatic degradation by serum ribonucleases and, due to this property, bypassing the phospholipid membrane to access the intracellular space.[3,176,177] In recent years, LNPs have gained tremendous and rapid translational value following their use in the Pfizer-BioNTech (BNT162b2, also known as Comirnaty®)[178] and Moderna (mRNA-1273, also known as Spikevax®)[176] vaccines against SARS-CoV-2. LNPs can be easily scaled up for mass production due to their relatively low cost and can be rapidly and inexpensively measured both *in vitro* and *in vivo* in a high-throughput manner.[179] Importantly, these LNPs can be formulated for long-term storage and easily modified to alter their properties such as stability, pKa, and clearance rate.[180]

LNP delivery of circRNA plasmids showed prolonged-expression and comparable initial expression efficiency compared to 5-methoxyuridine-modified linear mRNA both *in vitro* and *in vivo*.[181] The LNPs encapsulating circRNA constructed *in vitro* can be taken up by cells by endocytosis after injection.[182] In addition to their roles in the cellular uptake of circRNA, LNPs enhance endosomal escape, enable cytoplasmic delivery, and protect circRNA from degradation by endogenous nucleases.[182,183] In addition, LNPs protect circRNA molecules from being recognized in endosomes by toll-like receptors and retinoic acid-inducible protein I, thereby preventing over-activation of the innate immune system.[184]

Exosomes

Exosomes are nanoscale membrane-bound extracellular vesicles (EVs) that function as signalosomes, delivering messages in the form of bioactive molecules to specific recipient cells for intercellular communication.[185] Exosomes are inherently biocompatible with low immunogenicity and can overcome various biological barriers to deliver proteins, lipids and other biomolecules between different cellular environments.[186] Cell-derived exosomes are currently being explored as delivery vehicles for therapeutic endogenous circRNA targeting agents and expression vectors to exploit the natural functions of circRNAs in their respective disease pathways.[24]

In addition, exosomes can be used to directly deliver circRNA-expressing vectors. A landmark study demonstrated that engineered rabies virus glycoprotein-circSCMH1 EVs were generated to selectively deliver circSCMH1 to the brain.[187] These findings support that RVG-circSCMH1 EVs should be considered as a promising therapeutic strategy for functional recovery after stroke with great potential for clinical application.[187] Exosome-based carriers have been developed to deliver various therapeutic payloads, including short interfering RNAs, antisense oligonucleotides, chemotherapeutic agents and immune modulators, with the ability to direct their delivery to a desired target.[188] Exosomes are likely to be more biocompatible than synthetic nanoparticles, but their therapeutic use has also be challenged with safety and efficacy.[189]

Virus-like particles

Virus-like particles (VLPs) are self-assembling spherical nanoscale structures composed of viral structural proteins that lack viral genetic material and are therefore nonreplicating and noninfectious.[190] Their defined surface chemistry, uniform size and stability make them suitable and promising candidates for vaccination. In addition, the repetitive arrays on the surface of

VLPs are recognized by the immune system, eliciting strong humoral/cellular responses and providing an immunogenic delivery system for mRNA vaccines.[191-193] To date, at least 110 viral proteins from 35 viral families have been shown to assemble into VLPs.[194] From these studies, several VLP-based human vaccines, such as Gardasil®, Gardasil9® and Cervarix® against human papillomavirus,[195] have been licensed for clinical use and are now commercially available. Several other VLP-based vaccines, such as NVX-CoV2373 against SARS-CoV-2 virus,[196] are also in various stages of clinical trials.[197,198]

In addition to carrying peptides/proteins or other active molecules displayed on the surface of VLPs, they can be used as novel nanocarriers for targeted delivery applications.[199] Non-infectious VLPs carry various payloads, including chemotherapeutics or other drugs,[200] antibodies,[201] proteins,[202] siRNA,[203] contrast agents,[204] and even other nanoparticles.[193] Recently developed VLPs have emerged as potentially promising vehicles for gene editing agent delivery as ribonucleoproteins.[205] VLPs with circular mRNA show increased levels and duration of protein expression.[206] These studies demonstrate the versatility of VLPs through different loading strategies and multidimensional modifications, highlighting the potential of VLPs to deliver base editors and as promising vehicles for therapeutic circRNA delivery, leveraging the key advantages of both viral and non-viral delivery methods.

Viral vectors

Viral vector delivery can potentially be used to study the function and tissue-specific regulation of functional circRNA with viral tropism.[147] Recent studies have shown that the design and characterization of recombinant adeno-associated viral (AAV) vectors, which package transgene cassettes with intronic sequences that promote efficient back-splicing, generate circularized RNA transcripts.[147] *In vivo* circularization studies of the intronic elements known to be derived from the endogenous circRNAs ZKSCAN1 and HIPK3 provided the first proof-of-principle that artificial or naturally occurring circRNAs could be overexpressed using AAV vectors.[147] Subsequent studies have shown that many AAV vectors, with a wide range of host tissue tropisms and cell type specificity, allow delivery of the selected circRNA to multiple organ systems in various animal models and humans.[207,208] For example, researchers have used a recombinant AAV vector delivery system to overexpress or silence target genes to study the impact of the circSnd1/miR-485-3p/Olr1 axis on the progression of atherosclerosis.[207] Despite significant progress in the delivery of circRNA through the AAV capsid, several barriers still remain for the widespread adoption of AAV gene therapy, which includes anticapsid immune responses, the prevalence of neutralizing antibodies in the human population, low transduction rates in many therapeutically relevant cells and tissues, the inability to overcome physical and cellular barriers *in vivo*, and relatively limited carrying capacity.[209]

CIRCRNAS AS POTENTIAL BIOMARKERS

Recently, circRNAs have emerged as novel players in the onset and development of several human diseases. CircRNA profiles in biofluids are altered in a variety of diseases, suggesting that circRNAs may serve as circulating indicators of physiological status and targeted therapeutic tools in precision medicine.[210] CircRNAs have emerged as promising biomarkers and therapeutic targets due to their high stability and tissue or cell type-specific expression in various diseases.[211]

CircRNAs in immunological diseases

Immunological diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are associated with circRNAs. RA is one of the most common chronic polyarticular inflammatory diseases of unknown etiology. Several circRNAs in peripheral blood mononuclear cells (PBMCs), including hsa_circ_0025887, hsa_circ_0000396, hsa_circ_0001200, hsa_circ_0001566, and hsa_circ_0008360, have been validated and proposed as potential diagnostic biomarkers for RA.[212,213] SLE is another chronic autoimmune disease associated with widespread organ damage. Studies have revealed the potential of several circRNAs, including circPTPN22, hsa_circ_0044235, and hsa_circ_0045272, to serve as diagnostic biomarkers in SLE by analyzing the expression of circRNAs in PBMCs by high-throughput circRNA microarray or RNA-seq.[214-216] The mechanisms of these circRNAs may provide novel insights for clinical therapy of RA, SLE, and other immune-related diseases.

CircRNAs in cancer

CircRNAs are involved in the regulation of cell cycle, apoptosis, autophagy, angiogenesis, immune surveillance, invasion, and metastasis in cancers.[210] The functions of circRNAs in tumor progression may indicate the strong prognostic biomarker potential of these molecules.[217] In terms of prognostic biomarkers, ciRS-7 is the circRNA associated with poor prognosis in most cancer types.[218] Mechanistically, ciRS-7 promotes growth and metastasis in tumor cells via sponging of miR-7 and miR-876-5p, and their overexpression was associated with poor patient survival involved in colorectal cancer and esophageal squamous cell carcinoma.[219,220] CircFGFR1 has been proposed to function as an oncogene in non-small cell lung cancer (NSCLC), where patients with high levels of circFGFR1 have a worse prognosis than those with low levels.[221] Other individual circRNAs that have been described as tumor prognostic biomarkers include circLARP4 and circUBAP2. circUBAP2 has been described to function as a tumor suppressor, and circUBAP2 has been proposed as an oncogene associated with poor prognosis.[222,223] CircRNA panels and signatures have been shown to be more robust biomarkers for tumor prognosis. Researchers reported that a signature named cirScore, consisting of circPLOD2, circAGTPBP1, circISPD, and circPRKAR1B, could predict postoperative recurrence in stage II/III colon cancer.[224] CircSCORE is another signature based on 9 circRNAs that was highly predictive of time to progression in mantle cell lymphoma.[225]

CIRCRNA-BASED THERAPEUTIC STRATEGIES

CircRNA vaccines

CircRNAs are considered promising molecules in the field of neoantigen vaccines, and have the potential to become the next generation of RNA-based vaccine platforms, as they have shown therapeutic and prophylactic effects in COVID-19 and difficult-to-treat melanoma malignancies in 2022.[3,226] As synthetic circRNAs have shown superior stability, low immunogenicity, and enhanced protein production in mammalian cells, they can be used as an emerging application for therapeutic vaccination and drug delivery.[26,227]

A growing number of studies have demonstrated that engineered circRNAs can be used to express respective antigens to induce adaptive immune responses and therapeutic effects against disease.[3,228] For example, the circRNA^{RBD} vaccine elicited potent neutralizing antibodies and T cell responses by expressing the trimeric RBD of the spike protein, providing robust protection against SARS-CoV-2 in both mice and rhesus macaques.[3] Mechanistically, the circRNA^{RBD} vaccine enabled higher and more durable antigen expression and elicited higher surrogate IgG ratios of Th1-biased responses and increased proportions of neutralizing antibodies.[3] Another circRNA vaccine prototype, VFLIP-X, had detectable neutralizing antibody titers for up to 7 weeks post-boost against SARS-CoV-2 variants of concern and variants of interest and achieved a balance of Th1 and Th2 responses. Their results indicate that circRNA-delivered VFLIP-X induces humoral and cellular immune responses and broad neutralizing activity against the SARS-CoV-2 variant.[226] Despite these tremendous application prospects in recent years, the emergence and development of circRNA vaccines are still in the early stages. Only one clinical trial is currently enrolling healthy adult groups (number: NCT06205524) to evaluate the safety, tolerability and immunogenicity of a novel LNP-encapsulated circRNARBD vaccine. Further proof-of-concept clinical trials are required for the use of circRNAs as future vaccines.

CircRNAs can compensate for several disadvantages of canonical linear mRNA used in vaccines. First, circRNAs have excellent stability and RNase resistance. Their unique covalently closed structures prevent RNA degradation by exonucleases, providing high pharmaceutical stability and biostability compared to mRNA vaccines. Second, unmodified circRNAs are less cytotoxic and less immunogenic,[170] whereas mRNA vaccines have more side effects due to their higher immunogenicity.[229] Third, compared to mRNA, circRNAs have prolonged antigen delivery capabilities and sustained immune responses.[3] The resulting longevity and prolonged antigen production can effectively induce adaptive immune responses and generate more neutralizing antibodies.[3] Fourth, compared to unmodified and uridine-modified mRNA,

unmodified exogenous circRNA is able to bypass cellular RNA sensors and thereby avoid provoking an immune response in RIG-I and Toll-like receptor competent cells and in mice.[181] Finally, compared to mRNA vaccines, circRNA vaccines have properties that allow them to be produced quickly and economically in large quantities while being more stable and easier to store.[230] CircRNAs can be used to produce pandemic vaccine candidates within a few months or therapeutic tumor vaccines encoding neoantigens.[230] CircRNAs show great advantages over mRNA in protein translation and induction of anti-tumor immunity.[230]

Some remaining challenges in circRNA-based vaccines yet need to be addressed, such as increasing antigenic yields, improving circularization efficiency, ensuring adequate purification, and developing appropriate delivery approaches and doses, especially in clinical settings. Establishing effective delivery systems is crucial for enabling therapeutic applications. With further research and investment in this thriving field, these drawbacks are likely to be overcome in the near future.

CircRNA for protein replacement therapy

Protein replacement therapy can be defined as the transplantation of normal protein into cells to replace missing or defective protein with the goal of rescuing endogenous protein function with long-term expression and correcting genetic disorders or other diseases.[231] Modified mRNA can overcome the limitations of DNA-based or viral approaches to cardiac gene delivery and is increasingly being used in genetic medicine for protein replacement therapies and the treatment of genetic diseases.[232] In addition, *in vitro*-transcribed mRNAs using protein transduction domain technology have been developed for potential protein replacement therapy of the mitochondrial disease fatal infantile cardioencephalomyopathy and cytochrome c oxidase deficiency and β-thalassemia.[233] CircRNA protein replacement therapy offers several advantages over mRNA-based therapy, such as the ability to confer a more stable structure and allow for prolonged protein expression, developing inherently lower immunogenicity due to missing termini, comparable peak expression with a lower rate of decline, resulting in increased protein production and sustained protein expression levels.[231,234] Significant advances have been made in the *in vitro* synthesis circRNA with relatively long length, which facilitated circRNA used as a platform for protein replacement therapy applications.[170]

However, approaches based on the delivery of circRNAs that can be translated into proteins in target cells also face challenges. For efficient gene therapy *in vivo*, the delivery systems carrying the circRNAs must ensure: (1) uptake of the circRNAs by the target cells; (2) escape from the immune response; and (3) efficient translation and biodistribution of the circRNA. In recent years, considerable progress has been made in the use of LNPs to efficiently package and deliver circRNA molecules, promote endosome escape, and achieve robust circRNA translation *in vivo*.[3] However, their ability to efficiently integrate into the host genome, which provides the key benefit of sustained gene expression, also carries the risk of genome damage and tumorigenesis.[231] Most protein replacement therapies have focused on proteins that can be expressed in the liver cells, as most systemically administered LNPs reliably localize and accumulate in the liver.[235] Engineering of LNPs for efficient delivery of nebulized therapeutic RNA has been designed for the specific route of administration and type of drug, but results in lower delivery efficiency and is limited to pulmonary diseases.[236] Therefore, there is an urgent need to explore robust approaches to improve delivery efficiency and optimize the formulation of circRNA LNPs to address experimental and clinical needs in protein replacement therapies.

CircRNA for cancer immunotherapy

With the widespread use of cancer immunotherapeutic drugs and treatments, such as immune checkpoint blockade agents, chimeric antigen receptor (CAR)-T cells, and cancer vaccines, immunotherapy has received increasing attention in recent years.[237] Immunotherapy aim to better activate host antitumor immunity to create a tumor-suppressive microenvironment than chemotherapies and other agents, ultimately achieving tumor elimination and improving overall patient survival.[238]

The emerging functions of circRNAs in immune regulation give them great potential and advantages in cancer immunotherapy. The excellent stability of circRNA allows for the stable expression of protein therapeutics. Immune cells can be better recruited to the tumor site through prolongation of circRNA expression duration.[239] CircRNA encoding CAR can be customized through coding sequence modification to target different cancer types for precision cancer immunotherapies.[240] Cancer vaccines induce anti-tumor responses by expressing tumor antigens *in vivo*, enabling antigen-presenting cells to present and activate tumor antigen-specific T cells for a more robust immune response.[3] Vaccination of mice bearing breast cancer or melanoma with circFAM53B and its encoded peptides resulted in enhanced infiltration of tumor antigen-specific cytotoxic T cells.[241] Direct intratumoral delivery of circular mRNA encoding a mixture of cytokines successfully modulated intratumoral and systemic anti-tumor immune responses and enhanced anti-PD-1 antibody-induced tumor suppression in a syngeneic mouse model.[228] However, the use of circRNAs for cancer treatment in clinical practice is still a long way off. To date, only seven clinical trials on circRNAs and cancer have been initiated (accessed March 10, 2024).

Despite the tremendous progress in anti-tumor immunotherapies, there are still many issues that need to be addressed, in part due to tumor heterogeneity and side effects.[242] Overexpression of immunostimulatory cytokines and antibodies has resulted in significant dose-limiting toxicities.[243] For circRNA, prolonged protein production may increase the risk of harmful side effects. Therefore, there is an urgent need to control circRNA expression through switches and circuits[170] to encode specific proteins, prioritize the delivery of its payload to the tumor site, reduce the severity of side effects, and achieve safe circRNA-based cancer immunotherapy.[244] Current non-viral RNA delivery vehicles cannot selectively transfect cancer cells over healthy cells at clinical levels.[245] Further development of engineered circRNA using gene editing or intelligent delivery vector mechanisms to target tumor cells is needed for safer and more precise cancer immunotherapies.[240]

CircRNA for gene therapy

Gene editing has the potential to both improve our understanding of human genetics and treat genetic diseases and has motivated intense efforts to bring gene editing therapies into clinical practice. Several gene editing techniques, such as gene knockout, gene insertion, transcriptional regulation, and base editing, have been developed from this principle and are moving from bench to bedside.[246]

The CRISPR/Cas9 technique has been used to study the functions of cancer-associated genes, generate cancer models, validate druggable targets of essential genes, investigate drug resistance mechanisms, and explore non-coding gene regions. This has greatly expanded our understanding of cancer genomics due to its high accuracy and efficiency.[247] Base editing can repress circular and linear RNAs by targeting the splice sites involved in both back-splicing and canonical splicing.[157] Currently, base editing can be used to target approximately 95% of the pathogenic transition mutations in the ClinVar database[248,249] and is also being rapidly adopted to study various disease biology and treatments in animal models.[148,250] Prime editing is another important CRISPR/Cas-derived genome editing technique[160] and has been developed for targeted adenosine-to-inosine and cytidine-to-uridine conversions[161] resulting in consequent alteration of protein-coding sequences of selected genes. This technology has become a powerful tool for manipulating RNA to correct disease-causing mutations and modulate gene expression and protein function.[251]

Since gRNAs are easily degraded by nucleases and circular RNAs are theoretically immune to all RNA exonucleases, several circular gRNAs (cgRNAs) have been constructed and applied to the autocatalytic splicing mechanism of the RNA cyclase ribozyme.[172,252] CircRNA can express protein for longer periods, potentially leading to increased off-target effects. The Cas9 nuclease combined with the gRNAs will induce long-term expression and greatly increase the rate of off-target mutations, which can cause genomic instability and disrupt gene function.[253] Therefore, the reduction of the GC content and the shortening of the sequence length of the sgRNA used by modification of the corresponding Cas9 protein will reduce the off-target rate.[254] To improve editing efficiency and reduce off-target bystander editing, several advanced RNA base editors such as LEAPER 2.0,[255] CLUSTER[256] and circular ADAR-recruiting guide RNAs[172] have been generated in recent years. However, gene editing has so far been limited to the experimental stage due

to a number of challenges, such as delivery vectors and significant off-target editing.[257] Although prime editing has been successfully applied to cancer and primary cells,[258-260] patient-derived organoids,[261] zebrafish,[262] Drosophila,[263] bacteria,[264] and plants,[265] several key issues, such as lower editing efficiency and more complex gRNA design, remain to be addressed for prime editing to achieve the widespread applicability and therapeutic potential demonstrated by other precision genome editing technologies.[148,266] Immunogenicity may be another important barrier to the safety and efficacy of gene editing therapies. The expression of both circRNA and Cas9 in target cells can induce robust immune responses,[267-269] and the latter has been more easily packaged into viral vectors due to its smaller size.[270] Delivery of the Cas9 expression cassette to target cells by viral vectors can lead to long-term Cas9 expression *in vivo* and induce immune responses.[267] Given the less immunogenic nature of circRNAs, the therapeutic role of circRNAs also holds promise in avoiding adverse immune responses during editing.[28]

CONCLUSION

Discoveries in the field of circRNA biology have dramatically expanded our understanding of the major steps in circRNAs involved in disease development. As more research is conducted into the identification and circularization of circRNAs, their manipulation of expression levels, their delivery vehicles, and their therapeutic strategies, we will continue to better understand the precise and accurate characteristics of circRNAs.

CircRNAs have emerged as promising therapeutic targets due to their stability and tissue- or cell-type-specific expression. CircRNA is more stable than mRNA, making it suitable for long-term protein expression or target regulation. CircRNA is less immunogenic and safer than viral vector-based and DNA therapy, which can induce mutagenesis and high cytotoxicity. It is also easier to synthesize circRNAs and less expensive than viral vector-based therapy. The tissue-specific expression pattern of circRNA is necessary for the delivery of circRNA to specific organs or cell types in gene therapy, as it provides a precise and cost-effective method of circRNA treatment. To achieve the goal of therapeutic application in terms of specificity and efficacy, it is expected that new methods based on the specific expression characteristics of individual circRNA tissues will be explored in the future, i.e., systemic delivery of circRNAs but targeting specific organs for specific diseases. In conclusion, further understanding and profiling of tissue- and stage-specific expression patterns of circRNAs may lay the foundation for the therapeutic application of circRNAs.

Increasing evidence has shown that circRNAs hold great promise as diagnostic, prognostic, and predictive biomarkers. However, most biomarker studies lack adequate validation, which is critical for the clinical transformation of promising biomarkers. Specifically, internal and external validation cohorts with adequate and convincing power are needed to validate the generalizability of predictive models. Simultaneously, data from these prospective cohort studies must be comprehensive, reliable, large, consistent, and well-characterized.

Dysregulation of circRNA expression has been implicated in the pathogenesis of many diseases. By manipulating circRNA expression levels to intervene in disease progression, beneficial outcomes for patients can be achieved. Although altering the expression of circRNAs may enhance or expand their therapeutic power in various diseases, viral vectors expressing circRNAs may also cause unexpected side effects. Therefore, many obstacles remain in this field. In the coming years, studies to expand our understanding of the mechanisms of circRNA functions and to further develop specific and effective methods for targeting circRNAs *in vivo* will be key to increasing the clinical potential of circRNA-based therapies.

CircRNAs have yielded significant results in vaccine and nucleic acid drug development, such as a circRNA vaccine encoding SARS-CoV-2 RBD based on validation of good laboratory practices.[3,271] More importantly, the LNP-encapsulated circRNA-RBD vaccine dramatically induced potent, durable neutralizing antibodies, and its expression was not affected at room temperature for 2 weeks. Specifically, the circRNA vaccine has shown great potential in inducing antibodies that effectively neutralized the B.1.351 mutant strain in mice and for developing clinical applications against the SARS-COV-2 mutant strains.[3,223,271] Although current LNPs have achieved some success in translating RNA from bench to bedside, there is still a great need for improvement in targeting extrahepatic organs,[272] overcoming biological barriers,[273] enhancing endosomal escape,[274] and preventing unwanted immune activation.[273] To maximize the safety and delivery efficiency of circRNA vaccines, novel circRNA delivery systems are highly desired.

Emerging AI-based approaches facilitate identifying common and unique features of circRNAs, including their expression levels, alternative splicing patterns, co-expression networks, and unique structural motifs under different circumstances. AI simulates the chemistry between circRNAs and proteins and predicts the efficacy of circRNA drugs. In addition, AI can provide an algorithmic platform for circRNA structure prediction and sequence design, such as circDesign.[275] AI facilitates the circRNA drug design with multiple targets, provides synthetic routes, predicts reaction yields, and predicts synthesis mechanisms. Therefore, focusing on the implementation of AI technologies to advance the design of novel circRNA sequences and neoantigen prediction algorithms is a must to effectively accelerate the development of circRNA vaccines and other therapeutic uses.

In conclusion, the potential of circRNAs to make significant therapeutic impacts in the clinic may represent a robust platform for precision medicine. In addition, the clinical translation of circRNA-based therapeutic strategies to humans is of great theoretical value and clinical significance for precise disease diagnosis and treatment.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

Not applicable.

SUPPLEMENTAL INFORMATION

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LEAD CONTACT WEBSITE

Liang Chen: http://biomed.ustc.edu.cn/2023/0609/c31899a605469/page.htm
Ying Zhou: https://lcyx.ustc.edu.cn/2023/0615/c34248a606001/page.htm