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Review article

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Engineering circular RNA medicines

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Abstract

Circular RNAs (circRNAs) are a group of RNA molecules prevalent across various organisms and tissues and characterized by a covalent loop structure. Their unique structure, lacking 5′ and 3′ ends, confers resistance to exonucleases, thereby enhancing their stability compared to linear RNAs. Since the early 2010s, the versatility of circRNAs have been highlighted in applications such as RNA aptamers, guide RNAs and, more recently, SARS-CoV-2 vaccines. Recent advances in rational design, as well as in vitro and in vivo synthesis techniques, underscore the potential for large-scale engineering and production of circRNAs, positioning them as promising candidates for stable and efficient RNA-based therapeutics with minimal immunogenicity. This Review summarizes the guiding principles behind circRNA engineering and development, with a focus on key design elements. We also provide an overview of circRNA advances in disease prevention and treatment. By emphasizing existing limitations and outlining future milestones, this Review offers a translational outlook on circRNAs as an emerging field in biomedicine.

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Outlook

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Introduction

RNA-based therapeutics have emerged as a promising class of drugs owing to their scalability, programmability and potential for personalized medicine applications. Several RNA-based therapeutics, including antisense oligonucleotides[1], small interfering RNAs (siRNAs)[2], microRNAs (miRNAs)[3] and RNA aptamers capable of interacting with specific target proteins[4,5], as well as mRNAs encoding therapeutic proteins[6], have been granted FDA approval for clinical use[7]. However, linear-RNA-based therapeutics have several limitations, such as instability during storage and delivery owing to their susceptibility to degradation by endogenous and exogenous ribonucleases (RNases), along with the risk of inducing strong immune responses. Thus, RNA-based therapeutics need to be optimized to improve stability, reduce immunogenicity and increase their translational efficiency.

Circular RNAs (circRNAs) are a unique class of single-stranded non-coding RNAs characterized by their covalently closed loop structure that, unlike mRNA, lack the 5′ cap and the 3′ poly(A) tail. Generated co-transcriptionally via a back-splicing mechanism, wherein a downstream splice donor is joined to an upstream splice acceptor to form a closed loop, circRNAs can be found in various organisms and tissues[8,9]. They can act as microRNA sponges[10,11], interact with RNA-binding proteins (RBPs)[12], regulate gene expression[13] and even encode proteins[14], among others. Since the early 2010s, numerous circRNAs such as circORC5 (ref. 15), circNFIB[16], circRNA SCAR[17] and circFAM53B[18], have been found to contribute to the pathogenesis of various diseases, such as gastric cancer[15], intrahepatic cholangiocarcinoma[16] and nonalcoholic steatohepatitis (NASH)[17].

Owing to their distinctive circular structure, circRNAs are more resistant against exonucleases, with higher stability and prolonged half-lives[19] compared to linear RNAs. However, the production of circRNAs requires additional circularization and purification steps; designs are needed for more efficient circularization platforms while minimizing the introduction of foreign sequences that could trigger innate immune responses. CircRNAs can be generated in vitro using circularization techniques such as chemical ligation[20], enzymatic ligation[21,22] and permuted intron–exon (PIE) circularization[23–25], as well as in vivo approaches such as back-splicing and autocatalytic cleavage[26]. CircRNAs produced using these techniques can be leveraged as intracellular metabolite biosensors, vaccines targeting infectious diseases, therapeutics for genetic metabolic diseases and adenocarcinoma treatments. This Review summarizes the latest advances in circRNA design and manufacturing for different applications. Moreover, it provides an overview of circRNA-based vaccines and therapies with potential for manufacture scalability. Finally, we outline existing limitations and challenges and discuss how they can be overcome.

Engineering circRNAs

Endogenous circRNAs have a wide range of functions, including protein coding, microRNA sequestration, RBP interaction and regulation of parental gene transcription. This section describes circRNAs engineered for different purposes, such as therapeutic platforms (by encoding proteins) or *cis*-acting factors (Fig. 1).

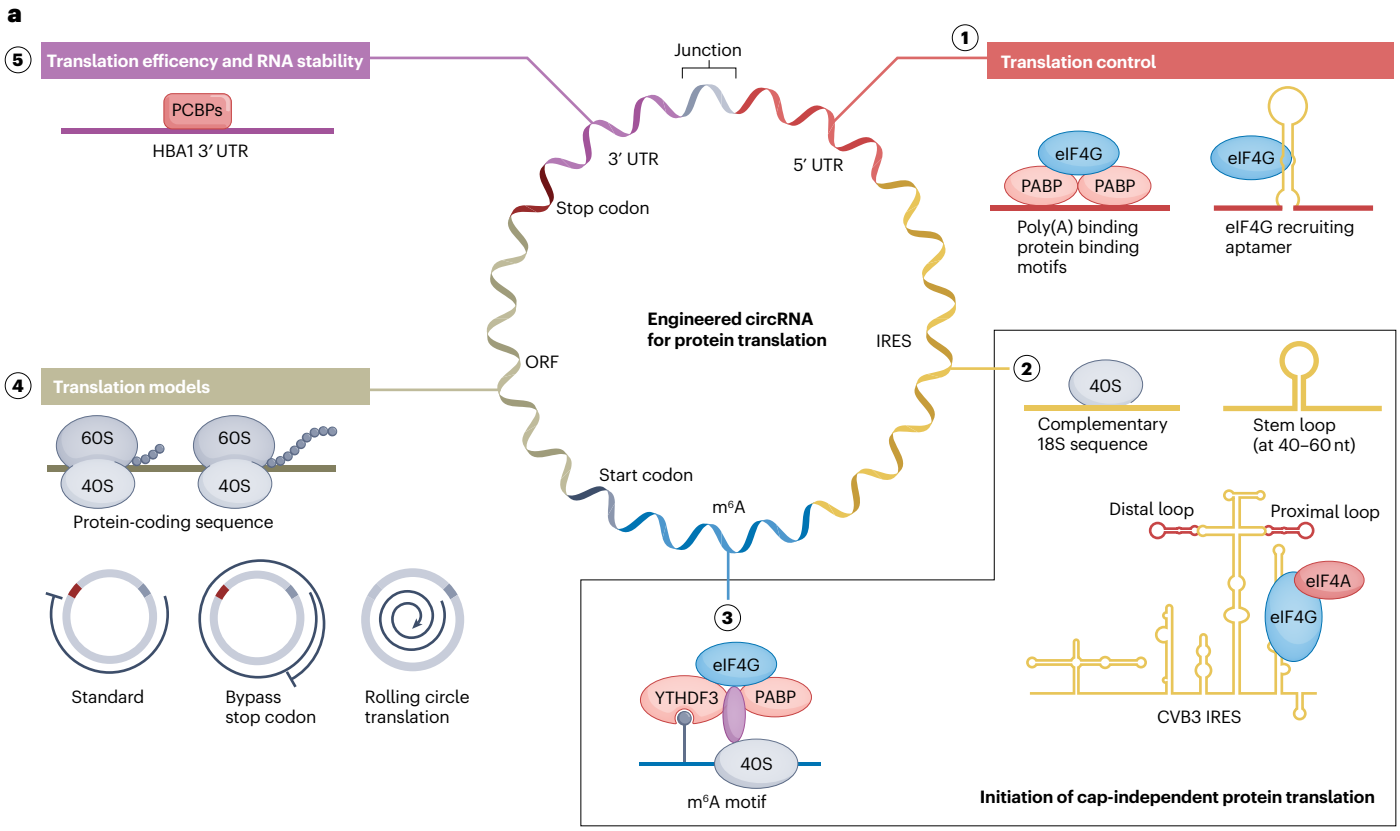
Protein translation

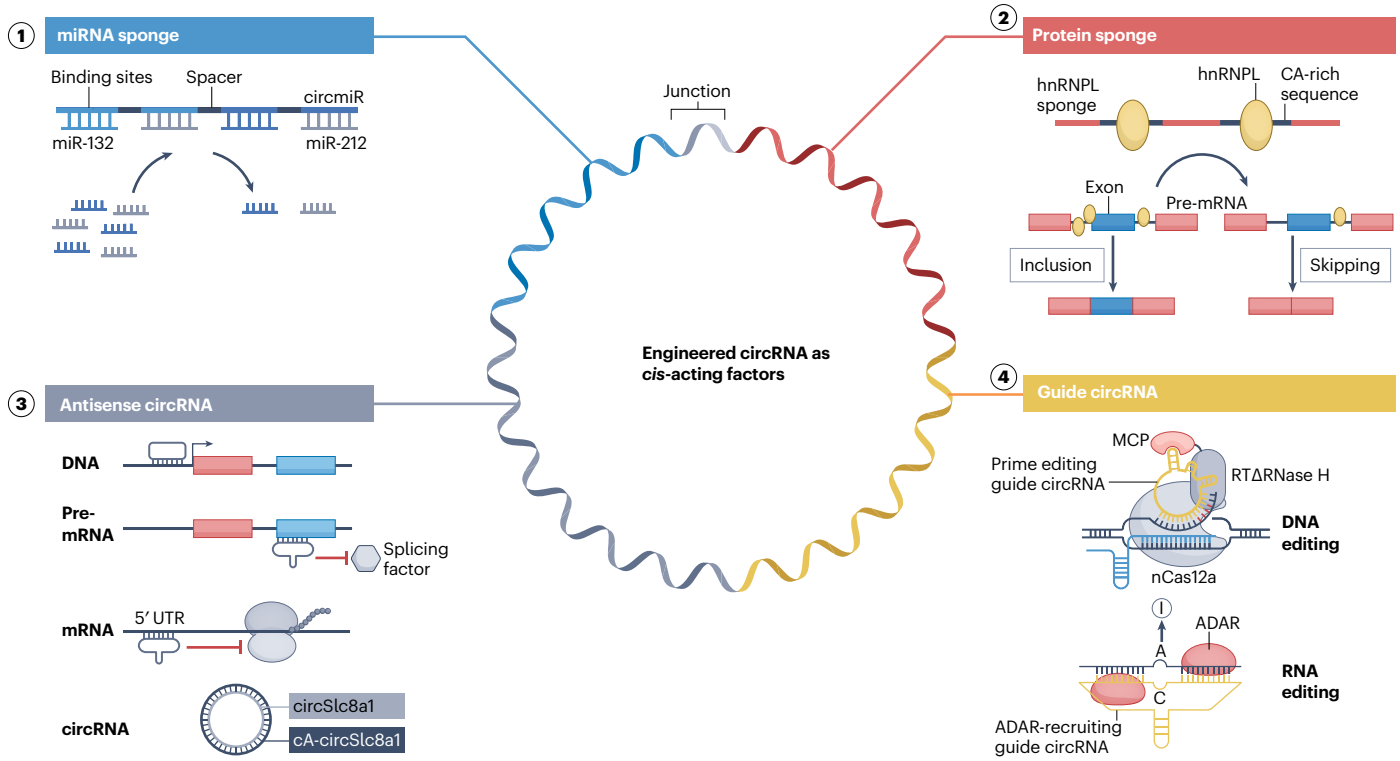
Traditionally, circRNAs have been classified as non-coding RNAs owing to their lack of the 5′-cap and the 3′-poly(A) tail required for cap-dependent translation[27,28]. However, using advanced high-throughput sequencing technologies, coupled with the development of sophisticated bioinformatics algorithms and rigorous biochemical validations, circRNAs that encode proteins in cap-independent mechanisms and exhibit active regulatory functions in human diseases have been discovered[14,18,29,30].

Owing to their high stability, circRNAs are suitable for encoding immunogenic and therapeutic proteins. Several strategies have been developed to engineer circRNAs into a safe and efficient protein-translation platform, such as the incorporation of 5′ untranslated regions (UTRs), internal ribosome entry sites (IRESs), N^6-methyladenosine (m^6A) motifs, open reading frames (ORFs) and 3′ UTR components.

Cap-independent translation initiation via IRES and RNA modifications. In eukaryotic cells, mRNA translation typically occurs in the cytosol and depends on the interaction of the 5′ m^7G cap and other translation-initiation factors[31] (Box 1). Despite lacking the 5′ cap structure, circRNAs can initiate translation through IRES elements in a cap-independent manner[32]. For example, a short 7-mer of 18S ribosomal RNA (rRNA) complementary sequences can initiate and enhance translation by directly base-pairing with rRNA and recruiting 40S ribosomal subunits[33] to drive active circRNA translation in HEK293T cells[34]. Furthermore, a 40–60-nucleotide (nt) compact stem-loop structured RNA elements within the domain II of the hepatitis C virus IRES promotes circRNA translation in a structure-dependent manner[34], without the need for IRES *trans*-acting factors (ITAFs) and translation-initiation factors (eIFs)[35]. By contrast, the translation initiation of extensively

Fig. 1 | Key components of engineered circRNAs for various purposes. **a**, Engineered circular RNAs (circRNAs) for protein translation. Design of a 5′ untranslated region (5′ UTR) to improve the translational efficiency of circRNAs. 5′ UTRs, including elements such as poly(A) binding protein (PABP) motifs and eukaryotic initiation factor 4G (eIF4G) recruiting aptamers (indicated in yellow), can increase circRNA translation by recruiting eIF4G, a key scaffold protein in the translation-initiation step (step 1). Incorporation of an internal ribosome entry site (IRES), such as CVB3 IRES, to initiate the translation process of circRNAs in a cap-independent manner. Short-IRES elements containing a complementary 18S sequence or a stem loop of around 40–60 nucleotides (nt) can also initiate circRNA translation. It has been reported that adding eIF4G recruiting aptamers at both the distal and proximal positions of CVB3 increases the translation efficiency of circRNAs (step 2). N^6-methyladenosine (m^6A) drives circRNA protein translation by recruiting the cytoplasmic m^6A reader YTHDF3, which interacts with the 40S and 60S ribosome subunits. m^6A modification abrogates circRNA immunity without activating the RIG-I innate immune signalling (step 3). A protein-coding sequence of circRNA includes a start codon, an open reading frame (ORF) and a stop codon. circRNAs encode proteins in different ways, including canonical linear ORF translation, stop codon bypass or rolling circle translation (step 4). Human α-globin 1 (HBA1) 3′ UTR sequence, which interacts with cytoplasmic poly(C) binding proteins forming an α-complex, has been reported to enhance circRNA translation efficiency (step 5). **b**, Engineered circRNAs based on sequence-based interference. The microRNA (miRNA) sponge, circmiR, was engineered with six miR-132 and six miR-212 binding sites with a 6-nt spacer between to act as miRNA inhibitors (step 1). Protein sponge, artificial heterogeneous nuclear ribonucleoprotein L (hnRNPL)-sponge circRNAs containing CA-rich sequence clusters modulate pre-mRNA slicing via sponging hnRNPL (step 2). Antisense circRNAs: through base-pairing, synthetic circRNAs can bind with DNA, pre-mRNA and mRNA to regulate activity (step 3). Guide circRNAs can be utilized in genome and transcriptome editing (step 4). ADAR, adenosine deaminases acting on RNA; MCP, MS2 stem loop coat protein; nCas12a, Cas12a nickase; RT, reverse transcriptase.


a
5 Translation efficency and RNA stability
PCBPs
HBA1 3′ UTR
Junction
1 Translation control
3′ UTR
5′ UTR
Stop codon
eIF4G
PABP
PABP
Poly(A) binding protein binding motifs
eIF4G
eIF4G recruiting aptamer
Engineered circRNA for protein translation
ORF
IRES
4 Translation models
60S
40S
60S
40S
Protein-coding sequence
Start codon
m6A
2
40S
Complementary 18S sequence
Stem loop (at 40–60 nt)
Distal loop
Proximal loop
eIF4A
eIF4G
CVB3 IRES
Standard
Bypass stop codon
Rolling circle translation
3
eIF4G
YTHDF3
PABP
40S
m6A motif
Initiation of cap-independent protein translation


b
1 miRNA sponge
Binding sites
Spacer
circmiR
miR-132
miR-212
Junction
2 Protein sponge
hnRNPL sponge
hnRNPL
CA-rich sequence
Exon
Pre-mRNA
Inclusion
Skipping
Engineered circRNA as cis-acting factors
3 Antisense circRNA
DNA
Pre-mRNA
Splicing factor
mRNA
5′ UTR
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circSlc8a1
cA-circSlc8a1
4 Guide circRNA
MCP
Prime editing guide circRNA
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DNA editing
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A
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RNA editing
ADAR-recruiting guide circRNA

Box 1 | Translation-initiation mechanisms

In eukaryotic cells, mRNA translation typically occurs in the cytosol and depends on the interaction of the 5′ cap and other translation-initiation factors[31,208]. This process involves recruiting ribosomal 40S subunits by translation-initiation factors (eIFs) and assembling the 80S ribosome complex at the mRNA start codon to initiate translation[31]. The cap-binding protein complex eIF4F, which consists of the decapping enzyme eIF4A[209], the cap-binding subunit eIF4F and the scaffolding protein eIF4G[210,211], is essential to recognize the 5′-cap and to recruit ribosomal 40S subunits.

Alternatively, translation can be initiated through internal ribosome entry sites (IRESs) elements in a cap-independent manner[32]. Over 1,000 IRESs have been discovered in viruses, human and other eukaryotic organisms[32]. Viral IRESs possess well structured RNA domains that hijack the host translation machinery for protein translation by interacting with IRES *trans*-acting factors (ITAFs) and eIFs[36]. By contrast, eukaryotic IRESs are less structured and less conserved, making it difficult to predict new endogenous IRESs in mRNAs[212]. Consequently, viral IRES mechanisms are better characterized, and most IRESs used to initiate circular RNA translation are derived from common viral IRESs.

Based on structural properties and the involvement of ITAFs[213] and eIFs[214] during ribosome recruitment, the IRES-mediated translation initiation mechanism can be further divided into three groups: (1) direct interaction with 40S subunits through ribosomal RNA complementary sequences or compact stem-loop structured RNA elements (SuRE); (2) interaction of ribosomal subunits and IRESs via eIFs (eIF4G and eIF4A); and (3) ITAF-dependent binding of eIFs, which subsequently recruits the 40S complex[214]. All these mechanisms have been leveraged for initiating circular RNA translation.

structured IRESs, such as the type I IRES from coxsackievirus type B3 (CVB3) and the type II IRES from encephalomyocarditis virus, can bind eIFs and ITAFs, thus driving stronger translation in circular RNAs[23,35–37].

Beyond native viral or endogenous IRESs, synthetic sequences have also been used to drive circRNA translation. For example, randomly pooled human rhinovirus (HRV) IRES fragments can initiate translation[37] and 93 shuffled HRV IRESs showed even stronger translational activity compared to wild-type iHRV-B3 IRES, suggesting that this strategy could be used to screen new IRESs with enhanced translational efficacy. By incorporating microRNA (miRNA)- and RBP-responsive switches, CVB3 IRES[38] and hepatitis C virus IRES[39] translation can also be tailored to selectively respond to the target miRNA or protein, which is suitable for targeted protein translation in cancer cells.

In addition to initiation by IRES, m^{6}A, the most abundant endogenous modification in eukaryotic RNAs, can also drive circRNA translation[40]. m^{6}A is typically identified at the consensus motif RRACH[41,42] (R = A/G, H = U/A/C, A represents methylation), with only one or two short motifs being sufficient to drive GFP translation of constructed circRNAs in human cells[40]. Notably, the well known mechanism of 5′ UTR m^{6}A-mediated translation by recruitment of eIF3 is 5′-end dependent, which is unlikely to mediate translation of circRNAs without 5′ and 3′ ends. Alternatively, m^{6}A-mediated circRNA translation involves direct recognition and binding of the m^{6}A modification by YTHDF3, which interacts with the initiation factor eIF4G2 and recruits ribosome subunits to initiate protein synthesis[40]. Numerous human circRNAs can be translated via this m^{6}A-mediated mechanism[40], probably because it is a universal mechanism given the widespread presence of m^{6}A in circRNAs[43]. Evidence showed that mutation of m^{6}A consensus sites in circE7 strongly suppresses E7 oncoprotein expression[44]. However, the exact translation mechanism initiated by m^{6}A is still unknown. In addition to its role in initiating the translation of circRNAs, m^{6}A modification functions as a marker for endogenous RNAs in mammalian cells. This modification can inhibit the activation of the innate immune system by hindering the recognition of exogenous circRNAs by RIG-I[45]. Notably, mutations in m^{6}A consensus motifs of in vitro synthesized circRNAs lead to an approximately two-fold increase in the induction of antiviral genes. Furthermore, exogenous circRNAs with just 1% m^{6}A modification substantially reduce immune responses in vivo[45], highlighting its potential in facilitating circRNA translation while minimizing immune reactions.

Different designs of circular open reading frames. Similar to linear mRNAs, the initiation of protein synthesis of most circRNAs with ORFs begins at the start codon (AUG) and terminates upon ribosome recognition of a stop codon (UAG, UAA or UGA) at the end of the ORF, yielding a protein identical to their linear counterparts. In addition, circRNA translation can initiate from non-canonical start codons CUG (23.2%), GUG (8.7%) and UUG (4.1%) within human mRNA ORFs[46]. For example, circZNF609 generates multiple protein isoforms using both AUG and non-AUG start codons in vitro[47,48]. Leveraging the unique circular structure of circRNAs, permutation of ORF sequences across the back-splice junction ensures that only the circular form, and not its linear counterpart, can generate the expected protein.

CircRNAs can also produce proteins longer than their ORF sequences[49]. If the length of the circRNA is not an integer multiple of 3, translation proceeds in a different frame after bypassing the back-splice junction, enabling continuous protein synthesis across the entire circRNA two or three times before termination at a frameshift stop codon[50,51]. For example, a 220-nt viroid circRNA with a UGAUGA motif (stop codon UGA, start codon AUG) produces a 16-kilodalton protein product using the frameshift mechanism[52]. In addition, rolling circle translation in the absence of a stop codon can be used to evaluate the efficiency of circRNA coding potential upon different initiation methods, whereby the generation of long repeating peptides from infinite circRNA ORFs can amplify the signal even with inefficient translation[48,53]. Overall, this design allows circRNA to encode proteins larger than the lenght of the circRNA, providing a flexible strategy for robust protein translation[54].

Other regulatory elements in the 5′ and 3′ UTR regions. The 5′ and 3′ UTRs are non-coding RNA sequences situated at the 5′ and 3′ flanking regions of the coding sequences. In mRNA, the 5′ and 3′ UTRs often contain regulatory elements that influence RNA localization, stability and translation. In general, the 5′ UTR contains upstream ORFs[55], IRESs miRNA or RBP binding sites, and other functional RNA structures such as stem loops[56], whereas 3′ UTRs contain adenylate–uridylate (AU)-rich elements and various protein binding sites[57,58]. These UTR elements do not directly contribute to protein synthesis but can influence the translation process by direct base pairing or establishing UTR-mediated protein–protein interactions[59] to the translation machinery.

Among the various regulatory elements, the Kozak sequence is a well-known 5′ UTR sequence (GCCACCATGG, with the start codon ATG)

that enhances start-codon recognition in eukaryotes[60]. The Kozak sequence has been widely used in mRNA translation, most recently in combination with the CVB3 IRES to initiate translation of circRNAs[49,61]. AU-rich elements are another important 3′ UTR regulatory element that enhances mRNA translation[57]; 97 IRES-like random hexamers capable of driving cap-independent circRNA translation can be clustered into 11 groups based on their AU-rich consensus motifs[53]. Moreover, a modular high-throughput platform has been used to evaluate the effect of different mRNA translation regulatory elements in promoting circRNA translation[37]. Notably, 5′ UTR poly(A)-binding protein motifs and the eIF4G-recruiting aptamer (Apt-eIF4G) can recruit eIF4G and promote the translation-initiation efficacy of the downstream CVB3 and iHRV-B3 IRES. At the same time, the human α-globin 1 (HBA1) 3′ UTR can also enhance the circRNA translation efficiency by interacting with cytoplasmic poly(C) binding protein to form an α-complex at their pyrimidine-rich *cis*-motifs[37,62]. However, not all elements of mRNA 5′ and 3′ UTRs have been used to promote circRNA translation[37]. For example, the 5′ UTR of mouse *Uchl1* mRNA forms high-order interactions through partial base-pairing with the *trans*-acting lncRNA *Uchl1AS*, which contains a SINEB2 repeat region that enhances ribosome binding and promotes translation[63]. Thus, similar high-order interaction mechanisms could potentially be engineered for circRNA translation. Future efforts aimed at exploring these regulatory mechanisms and identifying effective circRNA UTR elements may provide valuable insights for circRNA engineering, ultimately leading to more robust and efficient circRNA translation.

Cis-acting circRNAs

In addition to encoding proteins, circRNAs can also be engineered to act as *cis*-acting factors for other regulatory mechanisms (Fig. 1b).

microRNA sponges. The best known function of circRNAs is to act as microRNA sponges, where they competitively bind to microRNAs and upregulate the expression of downstream mRNA targets[10,11]. For example, the antisense strand of CDR1as (also known as ciRS-7), which contains over 70 binding sites for miR-7 (refs. 10,11), regulates the expression of target mRNAs such as RAF1, PAK1 and XIAP[64] by competitively inhibiting miR-7 activity, and has been implicated in brain development[65] and tumour progression[64]. Other miRNA-sponging circRNAs, such as circDYM[66], circORC5 (ref. 15), circMTO1 (ref. 67), cirPTK2 (ref. 68) and circFBXW7 (ref. 69) have also been identified in diseases affecting the brain, stomach, liver, lung and breast.

There is growing interest in engineering circRNAs as miRNA sponges[70]. For example, the engineered circRNA sponge scRNA21, which contains five repeated bulged binding sites for miR-21 can inhibit gastric cancer progression in vitro[71]. Similarly, circRNAs inhibit hepatitis C virus protein translation by sequestering miRNA-122s in vitro, thereby suppressing gastric cancer cell progression[72]. Another synthetic circRNA containing four miR-21-5p binding sites inhibited tumour growth in a lung adenocarcinoma xenograft mouse model[73]. To enhance miRNA binding efficiency, circmiR was engineered with six bulged binding sites for both miR-132 and six miR-212, separated by six nucleotide spacers[74]. Administration of adeno-associated virus (AAV) expressing circmiR resulted in attenuated cardiac hypertrophy in a mouse model of transverse aortic constriction[74].

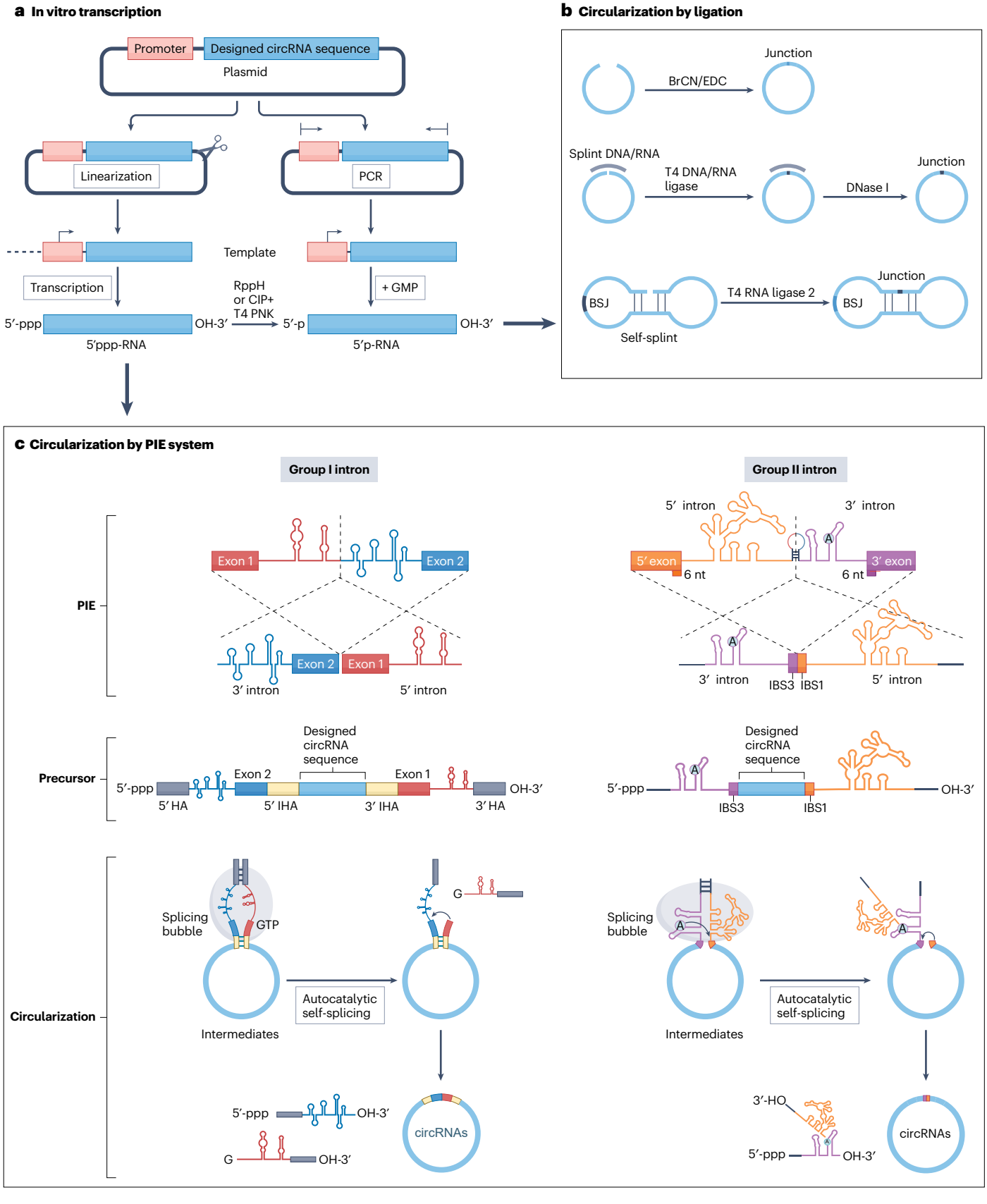
Notably, these circRNA sponges have a stronger effect than their linear counterparts[71–74]; for example, only 9% of circRNA sponges are degraded after 30 minutes of incubation in fetal bovine serum, whereas the corresponding linear sponges experience a degradation rate of 92%[71]. Moreover, in Huh-7.5 cells, circular sponges with bulged miRNA-122 binding sites have a stronger inhibitory effect on hepatitis C virus protein production by sequestering miRNAs more effectively than linear miRNA-122 sponges, especially when reducing the transfection amount of both circular and linear sponges by 80%[72]. However, challenges remain regarding the turnover stoichiometry of circRNA sponges and their target miRNAs, especially for endogenous circRNAs with limited copies and miRNA binding sites[75]. Thus, rigorous experimental validation and optimization of critical parameters, such as the number and type of miRNA binding sites and spacer sizes, are essential to ensure effective sponging.

Protein regulators. CircRNAs can directly interact with RBPs as protein scaffolds or antagonists, thereby post-transcriptionally regulating gene expression[76]. These interactions have been extensively studied using bioinformatic and experimental approaches[77]. For example, circVAMP3 is dysregulated in hepatocellular carcinoma; combining RNA pulldown, mass spectrometry and protein–protein interaction analysis revealed that circVAMP3 interacts with CAPRIN1 and G3BP1, which leads to the formation of stress granules to suppress the translation of MYC[78].

CircRNAs can be engineered to function as protein regulators for therapeutic applications; for example, direct transfection or overexpression of circRNA containing CA-rich high-affinity binding motifs inactivates heterogeneous nuclear ribonucleoprotein L (hnRNPL), with a similar effect to that of RNA interference (RNAi)-based knockdown[79]. The simultaneous nuclear and cytoplasmic localization of this circular CA-rich sponge resulted in more effective inactivation of hnRNPL compared to its linear counterpart[79]. However, the design of protein regulators requires rigorous screening for highly specific binding motifs because the same motif could be recognized by several proteins. For example, the binding of IMP3 to CA-rich sequences is similar to that of hnRNPL in this context[79].

***Cis*-acting regulator through direct base-pairing.** CircRNAs can also act as *cis*-acting regulators through direct base pairing with complementary RNAs or DNAs. One well documented mechanism involves exon–intron circular RNA (EIciRNA) regulating the expression of their parental genes in *cis* through direct RNA–RNA interactions[80]. For example, circEIF3J and circPAIP2 (ref. 80) bind to U1 small nuclear RNA and form ElciRNAs–U1–Pol II complexes at the promoter of their parental genes, thereby facilitating transcription[13]. Moreover, circSMARCA5 can interact with its host gene and form a circRNA R-loop structure at exon 15 to block transcription of SMARCA5 (ref. 81). Overexpression of circSMARCA5 enhances sensitivity to anti-breast tumour drugs in vitro and in vivo, indicating its potential as a therapeutic option for drug-resistant patients. Furthermore, intra-articular injection of lentivirus-circFOXO3 alleviates osteoarthritis by targeting its parental gene, *FOXO3*, and enhancing autophagy through activation of the P13K/AKT pathway in mice[82].

Similarly, synthetic antisense circRNAs (AS-circRNAs) have been developed to inhibit SARS-CoV-2 replication by targeting the structurally conserved 5′ UTR of the viral genomic RNA. In SARS-CoV-2-infected cells, AS-circRNAs exhibit stronger antiviral efficacy compared to modified linear AS-RNAs, largely thanks to their higher cellular stability[83]. This distinction might stem from differences in stability, subcellular localization, secondary structures and base-pairing potential between AS-circRNAs and linear AS-RNAs. However, the mechanisms contributing to the superiority of AS-circRNAs against SARS-CoV-2


a In vitro transcription
Promoter
Designed circRNA sequence
Plasmid
Linearization
PCR
Template
Transcription
+ GMP
RppH
or CIP+
T4 PNK
5′-ppp
OH-3′
5′ppp-RNA
5′-p
OH-3′
5′p-RNA
b Circularization by ligation
Junction
BrCN/EDC
Splint DNA/RNA
T4 DNA/RNA
ligase
DNase I
Junction
BSJ
T4 RNA ligase 2
BSJ
Junction
Self-splint
c Circularization by PIE system
Group I intron
Group II intron
PIE
Exon 1
Exon 2
Exon 2
Exon 1
3′ intron
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5′ exon
6 nt
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Precursor
Designed
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sequence
5′-ppp
Exon 2
Exon 1
OH-3′
5′ HA
5′ IHA
3′ IHA
3′ HA
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circRNA
sequence
A
5′-ppp
OH-3′
IBS3
IBS1
Circularization
Splicing
bubble
GTP
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Autocatalytic
self-splicing
Intermediates
5′-ppp
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circRNAs
Splicing
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Fig. 2 | In vitro generation of engineered circRNAs using ligation and the PIE system. a, Generation of linear precursors through in vitro transcription. DNA templates for in vitro transcription are derived from plasmids through either restriction enzyme digestion or polymerase chain reaction (PCR) amplification. These templates are transcribed by T7 or SP6 RNA polymerases under the control of T7 or SP6 promoters. Transcription in the presence of nucleoside triphosphates (NTPs) generates 5′-triphosphate RNAs (5′-ppp RNA), which can be used for group I/II intron circularization. For circularization by ligation, the 5′-ppp must be removed, typically by treatment with RNA 5′ pyrophosphohydrolase (RppH) or by using a combination of calf intestinal alkaline phosphatase (CIP) and T4 polynucleotide kinase (PNK) to leave a 5′ monophosphate (5′ p-RNA). Alternatively, 5′ p-RNAs can be generated in the presence of guanosine monophosphate (GMP) rather than GTP during transcription. **b**, In vitro circularization by chemical and enzymatic ligation. RNAs can be circularized by chemical ligation in the presence of condensing agents such as cyanogen bromide (BrCN) or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). In addition, T4 DNA/RNA ligase can catalyse the ligation of 5′-phosphate and 3′-OH ends of RNAs with the aid of a DNA/RNA splint. Recently, to reduce by-products during circularization, one strategy takes advantage of circular RNAs (circRNAs) own sequences to bring the ends of 5′p-RNA into proximity, eliminating the need for T4 DNA splints, and taking advantage of T4 RNA ligase 2 for ligations. **c**, In vitro circularization through the permuted intron–exon (PIE) systems. Engineered circRNA sequences are inserted into permutated group I or II introns. Homology arms (HA) are added to bring exons from the 3′ and 5′ ends into close proximity through base pairing, thus increasing circularization efficiency. Autocatalytic group I intron circularization then occurs in the presence of free GTP. The CirCode system represents an optimized self-catalysed group II intron PIE circularization system. The intron is split at the D4 domain (indicated by the green line), leaving only six nucleotides (nt) of the 5′-intron binding site (IBS1) and 3′-intron binding site (IBS3) after the self-catalysed splicing reaction, resulting in efficient production of circRNAs with the shortest sequence from the exon through a co-transcriptional mechanism. BSJ, back-splicing junction; IHA, internal homology arm.

remain unclear and warrant further investigation. Furthermore, AS-circRNAs can induce exon skipping by targeting the precursor mRNA both in vitro and in vivo[84]. For example, they restored dystrophin expression in a mouse model of Duchenne muscular dystrophy, highlighting their potential therapeutic utility in genetic diseases[84]. Moreover, a circular antisense RNA against circSlc8a1 (cA-circSlc8a1), generated from cA-circSlc8a1 plasmid by back-splicing and perfectly complementary to circSlc8a1, impaired the cardiac function of transgenic mice by blocking the cellular function of circSlc8a1 without influencing the *Slc8a1* mRNA[85]. This AS-circRNA is promising for gene therapy applications by silencing dysregulated circRNAs through the formation of double-stranded RNA circles.

Transcriptome and genome editing. Both transcriptome and genome editing have emerged as promising therapeutic strategies for the treatment of genetic diseases. Engineered circRNAs can overcome the low efficacy and short lifespan of linear guide RNAs in both genome and RNA editing. Specifically, engineered circular adenosine deaminases acting on RNA (ADAR)-recruiting guide RNAs (arRNAs) have been designed to recruit endogenous ADARs, enabling robust, persistent, and highly specific A-to-I RNA editing in vitro and in vivo[86,87]. Notably, circular arRNAs were more efficient than linear arRNAs, with sustained effects over time; after two weeks of injection, AAV-delivered arRNAs achieved 11% and 38% on-target editing in mice livers for single-copy and two-copy constructs, respectively. By contrast, no editing was detected for AAV-linear guide RNAs[83]. Moreover, arRNAs have 3.1 times greater efficiency than their linear counterparts, with editing effects lasting up to 21 days[87]. Engineered circular RNAs containing a reverse transcriptase template and a primer binding site have also been used as guide RNAs to develop circular RNA-guided CRISPR–Cas12a prime editors[88]. By arraying CRISPR RNAs targeting different genes in the 3′ region of one circular RNA, CRISPR–Cas12a prime editors were able to edit up to four genes simultaneously in human cell lines[88].

Synthesis and purification of circRNAs

Unlike linear mRNA molecules, circRNAs require additional circularization and purification steps, which need to be optimized to ensure high efficacy, purity and reduced immunogenicity. Different methods, including chemical ligation, enzymatic ligation and ribozyme catalysis, can be used for in vitro circRNA synthesis, followed by rigorous purification processes (Fig. 2). Alternatively, delivering plasmid DNA to target cells enables in vivo expression of circRNAs via mechanisms such as back-splicing or autocatalytic post-transcriptional cleavage (Fig. 3). This section offers an overview of strategies for in vitro and in vivo circRNA synthesis.

In vitro synthesis

The in vitro circularization of RNA involves the fusion of the 3′-OH and 5′-phosphate ends of linear RNA precursors transcribed in vitro[89], resulting in the formation of a closed circular structure with a covalent 3′–5′ phosphodiester bond. Several methods are used for circular RNA ligation, including direct ligation facilitated by chemical reagents or various ligases, as well as ribozyme-mediated self-catalysed ligation. Linear RNA precursors are typically synthesized using RNA polymerases within an in vitro transcription system consisting of DNA templates, ribonucleotide triphosphates, and RNA polymerases (for example, T7, SP6 T3 bacteriophage polymerase) along with specific reaction buffers[90], such as $MgCl_2$, spermidine and NaCl. The DNA template is prepared through PCR amplification or linearization from plasmids by restricting endonuclease digestion and must contain a transcription initiation sequence such as the T7 promoter for recognition by the RNA polymerase to initiate transcription. Ensuring that the first base on the linear RNA precursor is guanosine (G) substantially enhances transcription efficiency. The purity and concentration of the DNA template, as well as the reaction time, also have crucial roles in determining reaction efficiency.

Chemical and enzymatic ligation. Chemical and enzymatic ligation both require a linear RNA precursor with a 5′ monophosphate and a 3′-OH end[91]. However, in vitro transcription typically yields linear RNA products with a 5′ triphosphate, requiring subsequent steps such as RNA decapping using RNA 5′ pyrophosphohydrolase[92] or dephosphorylation by phosphatases, followed by rephosphorylation by T4 polynucleotide kinase[93]. Moreover, the addition of guanosine monophosphate to the in vitro transcription reaction can produce RNA with a 5′-monophosphate instead of the 5′-triphosphate, providing a substrate for downstream circularization processes[21].

Chemical ligation strategies use condensing agents such as cyanogen bromide (BrCN) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for RNA circularization[94]. However, these methods often result in side-reactions, resulting in unwanted 2′–5′ phosphodiester formation, which reduces ligation efficiency and raises

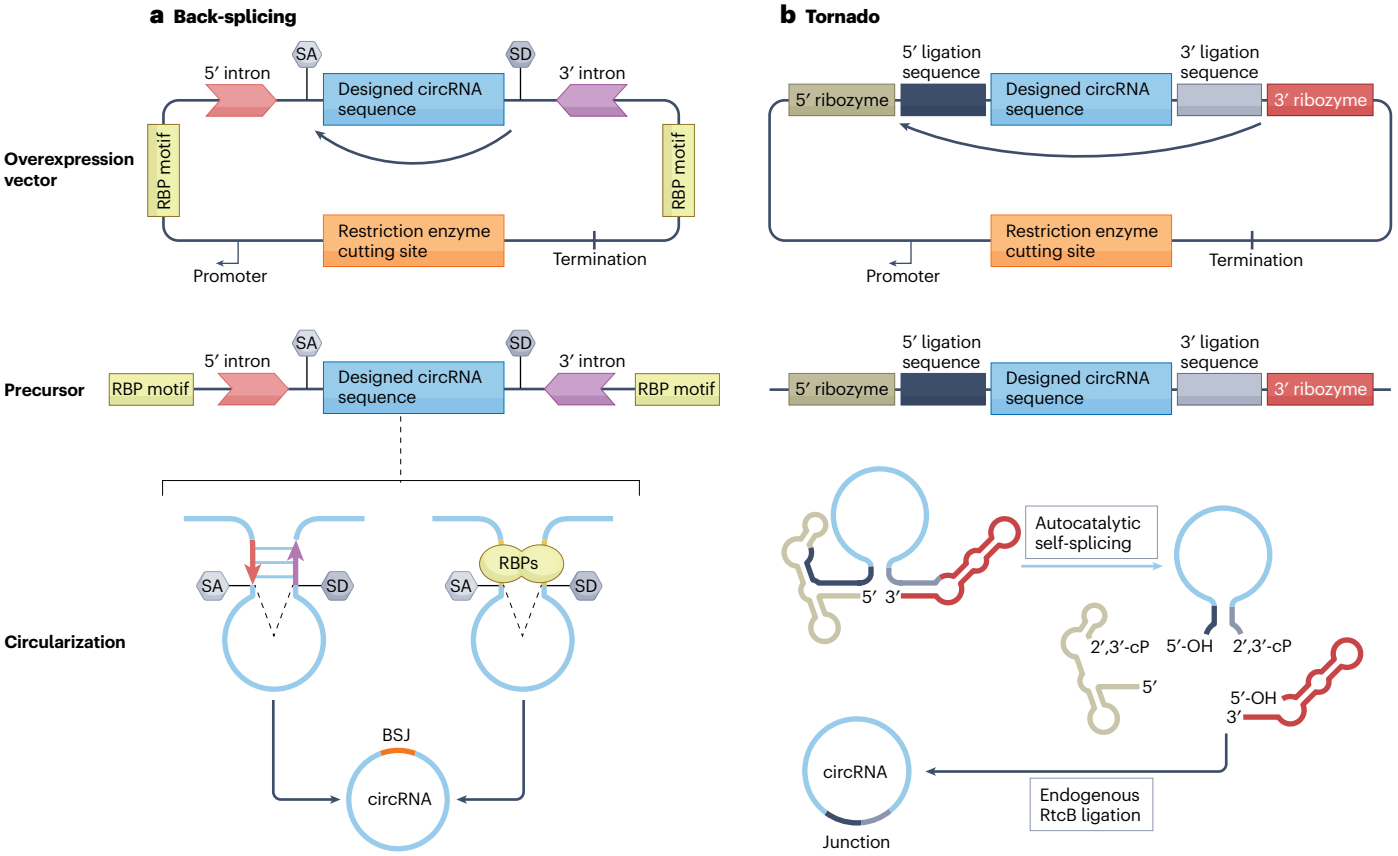

Fig. 3 | In vivo generation of engineered circRNAs. a, Circularization through the back-splicing mechanism. The engineered circular RNA (circRNA) sequence is inserted between base-paired introns in a constructed plasmid. After transcription, the circRNAs is generated by back splicing to form a back-splicing junction (BSJ) where the donor sequence (SD) covalently links with the acceptor sequence (SA). To enhance circularization efficiency, RNA binding proteins (RBP) motifs can be inserted adjacent to the back-splicing introns to recruit RBPs. **b**, Circularization using the Twister-optimized RNA for durable overexpression (Tornado) system. The designed circRNA sequence is inserted between 5′ and 3′ ligation sequences, which bring the 5′-OH and 3′-2′,3′-cP, generated by self-catalysed ribozymes, into proximity, allowing them to be ligated by endogenous RNA 2′,3′-cyclic phosphate and 5′-OH ligase (RtcB) in mammalian cells.

biosafety concerns. Thus, chemical ligation methods are currently not the mainstream approach for RNA circularization.

Enzymatic ligation can be achieved using T4 DNA ligase, T4 RNA ligases 1 and 2 (ref. 91), all of which catalyse the formation of 3′–5′ phosphodiester bonds between the 5′ phosphate and the 3′-OH in an ATP-dependent manner[91]. Typically, the ligation reaction requires that the termini of the RNA substrate are free of secondary structures. Thus, the addition of an approximately 20-nucleotide splint sequence prevents linear RNA folding and brings the circularization site closer, thereby improving efficiency. Notably, T4 DNA ligase recognizes nicks in base-paired double-stranded duplexes and relies on complete complementarity between the DNA splint and the linear RNA precursor[95]. However, its efficiency in joining DNA/RNA hybrids is over 1,000-fold lower than that of double-stranded DNAs[96], leading to RNA ligation rates that are 10 to 1,000 times lower compared to RNA ligases[97]. Consequently, this method is rarely employed for circular RNA synthesis.

By contrast, both T4 RNA ligases 1 and 2 exhibit higher RNA circularization efficiency. While the single-stranded RNA ligase (T4 RNA ligase) does not rely on DNA splint assistance, it operates with 100- to 1,000-fold lower efficiency than T4 RNA ligase 2 when working with double-stranded substrates. Meanwhile, the double-stranded RNA ligase T4 RNA ligase 2 exhibits higher ligation efficiency for double-stranded RNA substrates when internal complementary sequences[98] or guide RNAs are present. This ligase can achieve circularization of self-splinted RNA precursors with over 90% yield[98] and is widely used to produce circRNA vaccines and functional circRNA aptamers[61,99]. Overall, enzymatic ligation strategies do not involve exogenous nucleotides, thereby minimizing immunogenicity. However, challenges such as the high cost of enzymes, low efficiency of circularization for longer or highly structured RNAs and side reactions in intermolecular linkages still require further optimization.

Ribozyme-catalysed circularization. Group I and group II introns are two major classes of self-splicing ribozymes capable of catalysing their own excision from precursor RNA molecules without additional enzymes. Leveraging this ability, the PIE system was developed, by inserting exogenous sequences between two permuted half group I introns[100]. In the presence of magnesium ions, external guanosine-5′-triphosphate (GTP) sequentially attacks the splice sites of the 5′ and 3′ introns[101], leading to the production of a circularized exon through a

two-step transesterification reaction. To improve RNA circularization efficiency, the catalytic intron from *Anabaena* was substituted for the T4 bacteriophage intron. The 19-nucleotide homologous arms, and internal homology arms and spacers were inserted at both the 5′ and the 3′ flank regions to enhance complementarity[23]. These modifications substantially improved circularization efficiency, enabling in vitro synthesis of circRNAs up to 5 kilobases. This refined PIE method has been widely used to synthesize functional circRNAs, including circRNA vaccines[61,102] and controllable translated templates[38]. However, extraneous fragments in circRNAs introduced by PIE can provoke innate immune responses[103]. To reduce immunogenicity and improve scalability of RNA circles, a 27-nucleotide stem-loop from the anticodon arm of $tRNA^{Leu}$ was placed at the junction site[104]. The *Anabaena* transfer RNA $(tRNA)^{Leu}$-derived PIE has been used to generate circRNAs of various sizes (up to 7 kilobases)[104], expanding the potential for designing circRNA-based modalities.

Despite the efficiency, cost-effectiveness and convenience of PIE methods over enzymatic ligation, residual ribozyme sequences in circularized products might still induce potential immunogenicity[53]. To address this concern, the updated 'Clean-PIE' technique employs codon degeneracy to design dual-function codon combinations that encode the target protein while also serving as components of the PIE introns[105]. Compared to the original PIE system, this approach reduces the introduction of additional sequences into the circularized products, facilitating simpler circularization with potentially higher circularization efficiency, higher product purity, lower immunogenicity and sustained protein translation efficiency[23]. Moreover, the P1 helix structure of the *Tetrahymena* group I intron alone is sufficient to generate circRNAs without introducing extraneous fragments[24]. Similarly, the group II intron-based PIE can be used for in vitro circularization, whereby group II intron splicing occurs via two ester exchange reactions. During the autocatalysed splicing reaction, the 5′ splice site is first attacked by the 2′-OH of an adenosine residue in the intron. Then, the newly formed 3′-OH terminus residue attacks the 3′ splice site, generating a spliced exon–exon junction and a branched intron with a 2′–5′ phosphodiester bond[106,107]. Based on the PIE strategy, the circular coding RNA (CirCode) system has been developed for autocatalytic extracellular circularization using a permutated group II intron[25]. Through rational design and tailored modifications of group II intron sequences, CirCode achieved approximately 80% circularization efficiency, producing scarless circRNAs without extraneous sequences and extending their translational capacity. Collectively, these scarless circularization methods could mitigate potential immunogenicity concerns associated with the introduction of foreign sequences into circRNA.

Hairpin ribozymes provide an alternative approach for the circularization of short RNA molecules. Hairpin ribozymes belong to a class of self-cleaving RNA molecules characterized by a highly folded hairpin or stem-loop structure[108]. During cleavage, both the 3′ and 5′ ends are removed to form an intermediate containing a 5′ hydroxyl and a 2′,3′-cyclic phosphate, which is subsequently ligated into a circular form[109]. This circularization reaction primarily yields small RNA circles ranging from 50 to 150 nucleotides[110], and the process exhibits inherent instability owing to the dynamic equilibrium between catalytic cleavage and ligation. More recent controllable circularization systems involve an engineered hairpin designed to prevent self-ligation, coupled with additional RNA activators and polyamine co-factors[111]. Binding of the activator RNA and spermine co-factor induces a conformational change in the ribozyme, activating cleavage and circularization. Notably, the final circularization products are irreversibly stabilized upon separation of the activator from the circRNA.

Current in vitro strategies can achieve over 90% circularization efficiency and an approximately 90% circRNA yield after HPLC purification. Nonetheless, there is potential for further improvements; for example, chemical ligation efficiency can be further optimized using click chemistry methods such as azide–alkyne cycloadditions[20,112]. Moreover, enzymatic ligation could also be refined by using splint DNA with modified adenine analogue (2,6-diaminopurine)[113] and by screening or designing RNA ligases and ribozymes with improved catalytic activity[114]. Beyond focusing solely on circularization efficiency, unresolved biosafety concerns regarding the potential immunogenicity of in vitro synthesized circRNAs persist. Thus, comprehensive evaluation and clinical trials are essential to improve our understanding of these critical characteristics of circRNA therapeutics.

Enrichment and purification. Linear RNAs generated during in vitro circularization result in strong cellular immune response[115]. Enrichment and purification of circularized products are therefore essential to improve efficiency and mitigate immunogenicity. One approach involves the use of RNase R, a 3′–5′ exoribonuclease known for its ability to digest linear RNAs while preserving circular RNAs[116]. Nevertheless, effective digestion by RNase R requires a sufficiently long single-stranded 3′ overhang for initiation, and the presence of highly structured G-quadruplexes can impede RNase R digestion. To address this challenge, the substitution of Li^+ for K^+ has been proposed to destabilize the G-quadruplex structure, thereby increasing the digestibility of G-quadruplexes by RNase R[117]. However, prolonged reaction times of 15–60 minutes might inadvertently lead to nonspecific nicking of the circRNA[118], potentially compromising the purity of the final products.

Alternatively, size-dependent purification techniques such as polyacrylamide gel electrophoresis and high-performance liquid chromatography (HPLC) can be used to purify circRNA according to molecule size[23–25,103,115]. In particular, HPLC offers several advantages, including high selectivity, robust sensitivity and the capability for mass production with circRNA typically achieving purity levels over 90%[23,61]. Moreover, ion-pair reverse-phase HPLC and size-exclusion high-performance liquid chromatography could further improve the resolution required for separating smaller fragments with minimal differences in size[105,119].

In vivo expression

Intracellular circRNA synthesis involves mimicking natural circRNA biogenesis by transfecting DNA plasmids containing the designed sequences. After plasmid transcription, splice acceptor and donor sites are brought closer by reverse complementary sequences[23] or RBP binding motifs[120] in flanking introns. This proximity facilitates nascent circRNA generation via the back-splicing mechanism[121]. Complementary introns can achieve circularization of 92% of the total exogenous transcript in vivo, comparable to in vitro synthesis[122].

Circularization strategies such as the PIE and hairpin ribozyme systems do not require additional ligation factors and can also be used for in vivo circRNA expression. Additionally, the splicing of tRNA introns in Archaea and animals can produce tRNA intronic circular RNAs (tricRNAs), providing an alternative approach for in vivo circularization[123]. However, this method can result in high levels of uncleaved linear precursors that limit circularization effciency[124]. The Twister-optimized RNA for durable overexpression (Tornado) system uses an optimized twister ribozyme for autocatalytic cleavage, generating a target RNA

with 2′,3′-cyclic phosphate and 5′-OH, which is further ligated by the endogenous RNA ligase RtcB[124]. The Tornado method achieves rapid ribozyme-assisted RNA cleavage within 5 minutes and produces abundant target circRNAs in HEK293T cells, whereas the yield with PIE is lower. Tornado not only enables efficient circularization but also supports high expression levels, making it a widely used technique for the in vivo expression of circRNA aptamers and guide RNAs[86–88,124].

Nevertheless, the dependence of in vivo circRNA expression strategies on vector delivery and endogenous biogenesis mechanisms poses challenges for the purification of circRNA products from organisms like *Escherichia coli* during large-scale industrial production[125,126]. Current vector-based circRNA overexpression systems primarily use adenovirus[127], adeno-associated virus[128] and lentivirus platforms[129]. However, caution should be taken with adenoviral vectors owing to concerns about potential integration of vector DNA into the genome and induction of strong immune responses[130]. In addition, in vivo expression of engineered circRNAs lacks accurate dosage control, raising concerns about dose-dependent effects in clinical applications. Furthermore, the linear splicing products of circRNA expression plasmids can also encode proteins, leading to unintended byproducts[47] and skewed quantification of circRNAs[125]. A mutagenesis study using a dual tag strategy revealed that many of the translational signals originate from cryptically spliced linear transcripts rather than from circRNAs[48]. Thus, thorough evaluation of the circular-to-linear ratio[131,132] is essential to distinguish desired circRNAs-encoded proteins from those translated from linear splicing artifacts or degraded intermediates. Finally, precise subcellular localization of engineered circRNA therapeutics is critical to achieve their intended functions. However, the mechanisms underlying nuclear export and organelle transport of circRNAs are not fully understood, making it difficult to ensure correct subcellular localization at the target organelle[133–135]. These issues must be addressed to ensure effective in vivo expression of circRNA therapeutics.

CircRNA-based vaccine and therapeutics

The rapid advance of RNA vaccines and medicines, boosted by the COVID-19 pandemic, had led to the FDA approval of mRNA-based vaccines for clinical use. CircRNAs can also have broad spectrum applications in disease prevention, including cancer vaccines[18] and the treatment of various diseases such as hepatocellular carcinoma[67], nonalcoholic steatohepatitis[17]. This section provides an overview of the translational potential of circRNAs as both preventive and therapeutic interventions.

Infectious and cancer vaccines

Despite their success during the COVID-19 pandemic[136], broad application of mRNA-based vaccines and therapeutics remains constrained by factors such as low stability, limited duration of expression, and potential immunogenicity[137,138]. Linear mRNA requires additional nucleotide modifications to maintain stability, whereas circular RNAs, owing to the absence of 5′- and 3′- ends, resist ribonuclease digestion with a half-life even 2.5 times longer than their linear counterparts in mammalian cells[139]. Similarly, in blood samples, circRNAs have substantially longer half-lives (24.56 ± 5.2 hours) compared to mRNAs (16.4 hours)[19]. This higher stability facilitates efficient and long-lasting protein translation[23,37]; engineered circRNAs, when intraperitoneally injected in mice, can consistently produce proteins for up to one week. By contrast, linear mRNAs modified with N1-methylpseudouridine (N1Ψ), often used to enhance safety and efficacy, exhibit a rapid decline in protein production within 48 hours[37]. Using stable and long-lived circRNAs can also reduce the costs of storage and transport.

Moreover, circRNAs can be designed to avoid immune responses; circRNAs containing human intron sequences processed as endogenous circRNAs abolish innate immune signalling after in vitro trasfection[140]. Similarly, the circRNA SARS-CoV-2 vaccine (which encodes the trimeric receptor-binding domain on the spike protein) encapsulated by lipid nanoparticles (LNPs) induce a more robust immune response, and have higher efficacy and thermal stability than 1mΨ-mRNA and unmodified mRNA forms in mice and rhesus macaques[61]. Even after two weeks of storage at room temperature, circRNAs can encode receptor-binding domain antigens without detectable loss[61]. Similarly, in vitro circularized mRNA delivered by LNPs encoding the spike protein with five proline modifications (VFLIP-X) induced robust hormonal and cellular immune responses, offering protection against infectious SARS-CoV-2 variant isolates and pseudotypes in mice[141]. To further enhance immunogenicity, the LNPs were modified with mannose to increase stability during lyophilization, which is the most common and effective approach for long term storage and transportation of RNA medicine[142]. A similar approach can be used to target dendritic and B cells in the lymph nodes, resulting in a more robust antibody response in mice[142].

CircRNA-G vaccines expressing the rabies virus glycoprotein and delivered by LNPs with mannose modification (mLNP-circRNA-G) can similarly generate antigens to induce a more potent and durable antibody response compared to 1mΨ-modified mRNA-G in mice[142]. Moreover, mLNP-circRNA-G remained stable at 4 °C with no remarkable changes in particle size, zeta potential, encapsulation efficiency or IgG titre in mice up to 28 days[142].

CircRNA vaccines have also been designed to encode various epitopes of *Staphylococcus aureus* to protect against nosocomial and community infections[143]. The general design and delivery carriers of in vitro generated circRNA vaccines can be easily adapted for other infectious diseases; however, the ORF sequence encoding the antigen and its corresponding entry site (such as IRES), specific modification and regulatory sequences that drive translation initiation and enhance efficiency, need to be further investigated and optimized to improve the efficiency of antigen expression. Moreover, similar to linear mRNAs, the dosing cycle and effective dosage of in vitro circRNA vaccines should be carefully evaluated to ensure a strong immune response while minimizing side effects.

CircRNAs can also be designed for immunotherapy and cancer vaccine applications by expressing tumour antigens. For example, the circRNA$^{OVA\text{-}luc}$ vaccine, initiated by CVB3 IRES expressing chicken ovalbumin (OVA), was circularized in vitro using the PIE-based assay and encapsulated by LNPs composed of multi-armed ionizable lipid[102]. Tail-vein injection of circRNA$^{OVA\text{-}luc}$ triggered an immune response and induced antitumour effects resulting in tumour progression inhibition without any observable side effects in immune-desert orthotopic mice models of metastatic melanoma. Notably, the combination of circRNA$^{OVA\text{-}luc}$ vaccines and adoptive T-cell treatment in late-stage immune desert orthotopic mice provide superior antitumour efficacy than monotherapy and can completely suppress the late-stage immune exclusive tumours by enhancing the persistence of TCR T cells[102]. In terms of innate immune response and antitumour response, no substantial differences were observed between mice immunized by circRNA$^{OVA\text{-}luc}$-LNP complex and m1Ψ mRNA$^{OVA\text{-}luc}$-LNP. Similarly, circOVA cancer vaccines engineered with the viral IRES, HRV-B3 (to initiate translation) and a 5′ poly(A)-binding protein and HBA1 3′ UTR sequence

Table 1 | Therapeutic applications of engineered circRNAs

Targets	circRNA	Mechanisms	Cell lines	Animal models	Delivery platform	Ref.
Vaccine						
SARS-CoV-2 Delta and Omicron	circRNA$^{RBD\text{-}Delta}$	Encoding protein	HEK293T, NIH3T3 and Huh-7	Female BALB/c mice Male rhesus macaques	LNP	61
SARS-CoV-2 variants	circRNA VFLIP-X	Encoding protein	HEK293T and HEK293T-hACE2	Female BALB/c mice	LNP	141
SARS-CoV-2	mLNP-circRNA-RBD	Encoding protein	HEK293T, DC2.4 and BSR	ICR mice	mLNP	142
Rabies virus	mLNP-circRNA-G	Encoding protein	HEK293T, DC2.4 and BSR	ICR mice	mLNP	142
Staphylococcus aureus infection	circRNA vaccine against *S. aureus*	Encoding protein	–	–	–	143
Cancer vaccine	circRNA^{OVA}	Encoding protein	HEK293T and NIH3T3	Female C57BL/6J mice	LNP	102
Cancer vaccine	CART-circOVA	Encoding protein	B16 and HEK293T	Wild-type C57BL/6J (000664) mice	CARTs	144
Cancer vaccine	circFAM53B[a]	Encoding protein	MDA-MB-231, MDA-MB-468, T47D, MCF-7, SK-BR-3, MCF-10A, HEK293T, B16-F10, 4T1, EpH4-Ev, T2, Melan-a	Melanoma and breast cancer mouse models (Female BALB/c, C57BL/6, NOD/SCID mice)	–	18
Therapeutics						
KRAS mutant tumours	GSDMD^{ENG} circRNA-LNP	Encoding protein	SU-DHL-5, Pfeiffer, RPMI-1788, AsPC-1, HPAF-II and T98G, HIEC-6, HT29, NHC-H2228, HCC-827, U118-MG, NF1 ipNF95.6, SW480, hTRET-hPNE and HBEC3-KT	hCD34^{+} mice OT-I and OT-II transgenic mice	LNP	159
Solid tumours (GBM, ESCC, LUAD, HCC, GC, THCA and CRC)	circLIFR (hsa_circ_0072309)[a]	Regulating genes expression	KYSE150, KYSE30, SK-Hep-1, HCT-116, HEK293T	Lung and liver metastasis tumours mouse models (BALB/c nude mice)	–	163
Major depressive disorder	circDYM[a]	microRNA sponge	Primary mouse microglia and BV-2 cells	CUS and LPS induced depressive-like mouse model (C57BL/6J male mice)	–	66
Major depressive disorder	circDYM[a]	Binding protein	BV-2	LPS-induced depressive-like mouse model (C57BL/6J male mice)	–	129
Acute ischaemic stroke	circSCMH1	Binding protein	HEK293T	Photothrombotic stroke mice (Adult male C57BL/6J mice)	Extracellular vesicles	194
Glioblastoma	circ-FBXW7 (hsa_circ_0001451)[a]	Encode protein	U251 and U373	Xenograft brain tumour formation in female nude mice	–	195
Glioblastoma	circ-SHPRH	Encode protein	U251 and U373	Nude mice xenografts	–	196
Glioblastoma	circPINTexon2	Encode protein	293T, U251, A172, Hs683 and SW1783	Female BALB/c-nu mice	–	148
Glioblastoma	circular AKT3	Encode protein	293T, U373, U251, Hs683 and SW1783	Female BALB/c-nu mice model	–	197
Glioblastoma	circHEATR5B (circ_0054048)	Encode protein	293T, U87, U251, U373 and A172	Subcutaneous xenografts in nude Mice	–	149
Hepatocellular carcinoma	circRNA-5692	microRNA sponge	HCCLM3, Huh-7, HepG2, cervical cancer Chang liver cells, HEK293T, WR168 and LX-2	C57BL/6 nude mice	–	198
Hepatocellular carcinoma	circVAMP3	Binding protein	HEK293T, SMMC-7721, Huh-7, HeLa and A549	Female athymic BALB/c nude mice	–	78
Intrahepatic cholangiocarcinoma	circNFIB (hsa_circ_0086376)	Binding protein	HuCCT1 and RBE	Liver orthotopic-implantation models and lung metastasis models in BALB/c nude mice	–	16

Table 1 (continued) | Therapeutic applications of engineered circRNAs

Targets	circRNA	Mechanisms	Cell lines	Animal models	Delivery platform	Ref.
Therapeutics (continued)						
Hepatocellular carcinoma	circASH2	Binding protein	LO2, HCCLM3, HA22T, Hep-SK1 and Huh-7	Orthotopic xenograft model and metastasis model in BALB/C nude mice	–	151
Hepatocellular carcinoma	hsa_circ_0001727 (circZKSCAN1)[a]	Encoding protein	HCC-LM3, SNU-398, Hep3B, Huh-7, SNU-387, HEK293T, THL-2	Murine and xenograft assay in female BALB/c nude mice	–	14
Nonalcoholic fatty liver disease	circRNA_0001805	microRNA sponge	Primary human hepatocytes	NAFLD model in Male C57BL/6	GA-RM/GZ/PL	178
Nonalcoholic steatohepatitis	circRNA SCAR	Binding protein	Primary human and mouse liver fibroblasts	High-fat-diet-induced cirrhosis in male C57BL/6J mice	mito-NP	17
Gastric cancer	circFAT1(e2) has_circ_0001461	microRNA sponge in cytoplasm Binding protein in the nucleus	HEK293T, GSE-1, SGC-7901, BGC-823, MKN-28, AGS, MGC-803 and MKN-45	Tumour formation assay in male BALB/c mice	–	199
Gastric cancer	m^6A-circORC5 (hsa_circ_0007612)	microRNA sponge	GSE-1, SGC-7901, BGC-823, MKN-28, AGS and MGC-803	Tumour-formation assay in male BALB/c mice	–	15
Gastric cancer	circDIDO1	microRNA sponge	MGC-803 and HGC-27	Xenograft model in nude mice	RGD-modified exosomes	155
Gastric cancer	circURI1	Binding protein	AGS and SGC7901	Tumour metastasis Assay in male nude mice	–	153
Gastric cancer	circMAPK1 (hsa_circ_0004872)	Encode protein	BGC-823, SGC-7901, MGC-803, MKN-45, HGC-27 and AGS	Tumorigenesis and metastasis model in nude mice	–	152
Gastric cancer	hsa_circ_0061137 (circDIDO1)	Binding protein Encode protein	SGC-7901, HGC-27, MGC-803, AGS and MKN-45	Subcutaneous xenograft tumour model in BALB/c nude mice	–	154
Gastric cancer	circGSPT1	Encode protein	293T, AGS, GES-1, SGC7901, BGC823, NCI–N87 and HGC27	–	–	156
Colorectal cancer	circFNDC3B[a]	microRNA sponge	LoVo, SW480, SW620, HCT116, FHC HUVEC and HEK293T	CRC tumour generation model in BALB/C nude mice	Exosome	200
Colon cancer	circFNDC3B[a]	Encode protein	NCM 460, DLD1, HCT116, SW480, LoVo, Caco2 and HT29	Xenograft mouse model in BALB/C nude mice	–	201
Colorectal carcinoma	circPLCE1	Encode protein	HCT8 (WT p53, WT KRAS, mutant APC), DLD1 (mutant p53, mutant KRAS, mutant APC), HIEC-6, NCM460 and HEK293T	Orthotopic xenograft CRC model and patient-derived xenograft model in NOD-SCID mice	–	202
Colorectal cancer	circMAPK14 (hsa_circ_0131663)	Encode protein	SW480, DLD-1, LoVo, HT29, HCT116, Caco2 and NCM460	Subcutaneous tumour model, lung metastasis model, liver metastasis in BALB/C nude mice	–	203
Myocardial infarction	circFndc3b[a]	Binding protein	Mouse cardiac endothelial cell line	Post-myocardial-infarction mice	–	204
Diabetic cardiomyopathy	circRNA DICAR (hsa_circ_0131202)	Binding protein	Heart tissue from mice	C57BL/KsJ WT and C57BL/KsJ *db/db*, $DICAR^{+/-}$ and $DICAR^{Tg}$ mice	–	127
Breast cancer	circSEMA4B hsa_circ_0000650	microRNA sponge Encode protein	HEK293T, MDAMB-231, HCC-1937, BT549, MCF-7, SKBR3, MCF-10A	Xenograft tumour assay in athymic nude mice	–	29
Breast cancer	circSMARCA5	Regulate host gene	293T, MCF-10A, MCF-7, SKBR3, BT474, T47D and MDA-MB-231	Xenograft tumour assay in female BALB/cnu/nu mice	–	81

Table 1 (continued) | Therapeutic applications of engineered circRNAs

Targets	circRNA	Mechanisms	Cell lines	Animal models	Delivery platform	Ref.
Therapeutics (continued)						
Triple-negative breast cancer	circFBXW7 (hsa_circ_0001451)[a]	microRNA sponge Encoding protein	MCF-10A, MCF-7, T47D, BT474, SKBR-3, MDA-MB-453, MDA-MB-468, MDA-MB-231, BT549, HCC38, 4T1 and MA-891	Xenograft tumour assay in BALB/c nude mice	–	69
Non-small-cell lung cancer	circPTK2 (hsa_circ_0008305)	microRNA sponge	BEAS-2B, A549, H1299, H1650, SPC-A1, Calu3, H226, H520 and SK-MES-1	Metastatic Mice model in female BALB/c nude mice	–	68
Non-small-cell lung carcinomas	circPTPRA	microRNA sponge	H23, H1755, H522 and BEAS-2B	Xenograft model in BALB/c mice	–	157
Lung adenocarcinoma	circASK1 (hsa_circ_0007798)	Encode protein	A549, HCC827, NCI–H1975, NCI–H1993, NCI–H1650, SPCA-1, SKLU-1 and H1993-GR	Xenograft model in BALB/c mice	–	158
Renal cell carcinoma	cRAPGEF5	microRNA sponge	769-P, Caki-1, OSRC-2 and 786-O	RCC-bearing xenograft and lung metastasis model in BALB/c nude mice	–	160
Diabetic nephropathy	circRNA_010383	microRNA sponge	SV40 MES 13, mTECs	*db/db* mice and *db/m* mice	–	205
Bladder cancer	circFNDC3B[a]	microRNA sponge	T24, UM-UC-3 and SV-HUC-1	Tumour xenografts in female BALB/c nude mice	–	206
Bladder cancer	circSLC8A1	microRNA sponge	5637, T24, J82, EJ, UMUC, RT4 and SV-HUC-1	Tumour xenografts in female nude mice	–	207

CART, charge-altering releasable transporter; circRNA, circular RNA; CRC, colorectal cancer; ESCC, oesophageal squamous cell carcinoma; GA-RM/GZ/PL, a metal–organic framework nanocarrier coated with galactose-modified red-blood-cell membrane; GBM, glioblastoma; GC, gastric cancer; HCC, hepatocellular carcinoma; HUVEC, human umbilical vein endothelial cell; LNP, lipid nanoparticle; LUAD, lung adenocarcinoma; mLNP, mannose-LNP; mTEC, medullary thymic epithelial cell; RBD, receptor-binding domain; THCA, thyroid cancer; WT, wild type. [a]Multifunctional circRNAs across different diseases.

(to enhance the translation efficiency) activate dendritic cells and trigger a T-cell response when delivered by charge-altering releasable transporters in a mouse model of melanoma[144].

Notably, the antigenic peptide circFAM53B-219, encoded by the endogenous circRNA, circFAM53B, from patients with breast cancer can serve as tumour-specific antigens to induce strong antitumour immune responses and inhibit tumour growth in mouse models of melanoma and breast cancer[18]. Moreover, vaccinated mice with natural tumour-specific circFAM53B also show antitumour resistance via tumour-specific T cell activity triggered by the antigenic peptide circFAM53B-219 (ref. 18). However, in vivo translation of the antigenic peptide circFAM53B-219, which is initiated by the native IRES from circFAM53B, can be hindered by the complexity of the physiological environment[18]. Thus, whether exogenous IRESs and translation regulatory elements can be used to construct circRNA cancer vaccines that encode endogenous tumour-specific antigen with higher translation efficiency and antitumour response needs to be investigated.

These and other examples highlight the potential of circRNA for preventive vaccine applications; however, most studies are still in the design or preclinical testing phase. Besides acting as vaccines, mRNA-based technologies have also been developed for protein-replacement therapy, targeting specific protein deficiencies caused by rare diseases[145]. For example, intravenous infusion of LNP encapsulated mRNA encoding human phenylalanine hydroxylase (PAH) leads to the production of the functional PAH protein in the liver of *Pah*^{enu2} mice, effectively restoring phenylalanine metabolism without any adverse clinical signs[146]. Notably, an mRNA replacement therapy targeting vascular endothelial growth factor for heart failure has successfully progressed from preclinical testing to the clinical stages (NCT03370887)[147].

CircRNAs as potential therapeutic targets

The widespread presence of circRNAs in various organs have sparked interest in harnessing them as therapeutic agents. Restoration of downregulated circRNAs can modulate disease progression in vital organs such as brain[129,148–150], liver[14,78,151], stomach[15,152–156], breast[29,69,81] and lung[68,157,158] (Table 1). For example, circHEATR5B, which is downregulated in human glioma tissues, includes an ORF that encodes the 881-amino-acid (aa) protein HEATR5B-881aa, which has tumour-suppressive activity[149]. Overexpressing OV-HEATR5B-881aa plasmids in glioblastoma multiforme cells inhibits the corresponding xenografts and extends the survival of nude mice[149]. In hepatocellular carcinoma, the nuclear localized circASH2 functions as a scaffold for complexes with YBX1, hnRNPs and tropomyosin4 (TPM4) transcripts, resulting in their degradation and, in turn, metastasis inhibition by altering the tumour cytoskeleton's structure in vitro and in vivo[151]. The circORC5 is methylated by the m^{6}A writer METTL14, which impedes gastric tumour progression by sponging miR-30c-2-3p, which in turn regulates the AKT1 expression in vitro and in vivo[15].

Another strategy is to engineer an oncolytic GSDMD^{ENG} circRNA that incorporates HRV2 IRES to initiate tumour-specific translation. The HRV2 IRES requires the assistance of initiation factor (eIF4G2) and ITAF (PTBP1) that are highly expressed in cancer cells to ensure high targetability and low levels of non-specific toxicity[159]. The protein encoded by GSDMD^{ENG} circRNA is equipped with a C-terminal mitochondrial signal peptide sequence that directs it to mitochondria, activating mitochondrial lytic properties and inducing antitumour immunity in mice[159]. Furthermore, weekly prophylactic intraperitoneal injections of GSDMD^{ENG} circRNA-LNPs reduce the incidence of adenocarcinogenesis induced by the *Kras*^{G12D} mutation in LSL-*Kras*^{G12D}/*Trp53*^{R172H} mice[159]. Using tumour-specific HRV2 IRES and intracellular organelles which

Table 2 | Industrial examples of circRNA

Company	Launch of circRNA biotech development	Location	Pipelines
Geneseed	2010	China	circRNA contract research organization
Circio	2010	Sweden	Cancer gene therapy[D] Vaccines[D] Rare disease[D] Mutant KRAS[C]
NuclixBio	2017	South Korea	Delivery
ORNA Therapeutics	2019 2024 (acquisition of ReNAgade_ Delivery)	USA	B cell-driven autoimmune diseases[D] B cell malignancies[D] (partner with Simnova) Sickle cell diseases[D] β-Thalassemia[D] Infectious diseases and others[UN] (partner with Merck)
Chimerna Therapeutics	2020	USA	Autosomal-dominant polycystic kidney disease[D] Alzheimer disease[D] Parkinson disease[D]
Circular Genomics	2021	USA	Precision psychiatry treatment[UN]
CirCode	2021	China	Vaccines (CC2206[D], CC2301[D]) Therapeutic proteins (CC2107[PC], CC2206[D], CC2301[D], CC2202[D])
Therorna	2021	China	Infectious diseases (one[PC], two[D]) Metabolic diseases[D] Cancer[D]
SYTE.bio	2022	USA	Therapeutics[D]
Ginkgo Bioworks	2022 (acquisition of Circularis)	USA	Colorectal cancer[UN] (partner with Esperovax[D] and Pfizer)
ORBITAL Therapeutics	2023	USA	Autoimmune disease and oncology[UN] Vaccines[UN] Protein therapeutics via RNA medicine[UN]

C, clinical; circRNA, circular RNA; D, discovery; PC, preclinical; UN, unknown.

target signal peptides present a new approach in designing precise and efficient circRNA therapeutics.

Notably, the same circRNA might function differently across different cells or diseases, raising safety concerns for the clinical application of circRNA-based vaccines or therapeutics. For example, circRNA cZNF532 (circBase ID: hsa_circ_0001681) inhibits renal cell carcinoma proliferation and migration through a microRNA sponging mechanism[160] while contributing to ferroptosis resistance in endometrial cancer through interaction with the RBFOX2 protein[30]. Similarly, hsa-circ-0000437 encodes peptides to suppress angiogenesis in endometrial cancer[161] while having an oncogenic role in gastric cancer progression[162]. The circLIFR (hsa_circ_0072309) also suppresses lung and liver metastasis in mice[163] and is involved in the downregulation of several other cancer types such as breast[164], glioblastoma multiforme, oesophageal squamous cell carcinoma, lung adenocarcinoma, thyroid, colorectal, gastric[165], hepatocellular carcinoma[166] and bladder cancer[167]. However, circulating circLIFR can also promote brain metastasis via the miR-100/ACKR3 pathway in a mouse model of non-small-cell lung cancer brain metastasis[168], raising safety concerns for its clinical application.

We anticipate that circRNA-based vaccines for infectious diseases will be among the first to transition from the laboratory to industrial-scale production. First, the key elements for in vitro production and in vivo translation of circRNA vaccines, including IRESs that drive translation and the regulatory elements that improve translation efficiency have been extensively characterized[34,37]. The pioneering SARS-CoV-2 (refs. 61,141) and RABV[142] circRNA vaccines can also be used as templates to shorten the development cycle and reduce costs. Moreover, advances in full-length circRNA sequencing strategies[169,170] and AI-based bioinformatic algorithms could promote the rational design and optimization of antigen-encoding circRNAs, potentially addressing safety concerns related to endogenous circRNAs that may exhibit varying functions across different diseases. For example, an RNA language model has been used to identify unannotated IRES and optimize 5′ UTRs for mRNA translation[171]. Moreover, deep generative models have facilitated the design of new functional ribozymes[172], a strategy that could be extended to improve circRNA stability and translation efficiency. However, challenges persist owing to the limited number of experimentally validated circRNAs available for model training.

Outlook

Engineered circRNAs have been used for various applications, including protein encoding, sponging of microRNAs and proteins, regulation of gene expression and guiding DNA/RNA editing. Design refinements and optimized in vitro and in vivo production methods have made circRNAs versatile candidates for therapeutic applications. Despite the growing interest and substantial investments in circRNA-based medicines, the industry is still in its early stages, with no approved circRNA therapeutics for clinical applications (Table 2). Clinical translation and widespread use of circRNA medicine will depend on its cost-effective mass production, which in turn requires optimization of circRNAs manufacturing steps, including circularization and purification, which are often complicated. To address these limitations, further in silico optimization to identify suitable ribozymes could improve circularization efficiency and support large-scale industrial production. AI models may also assist in discovering elements that enhance durable circRNA translation.

Therapeutic applications require delivery to the desired diseased organ, tissue or even intracellular organelles[173]. Given the cell-specific expression[174,175] and multifunctionality of circRNAs across tissues, a robust delivery system is crucial for circRNA therapeutics to circumvent the in vivo immune response[176] and to shield against degradation mechanisms[177]. However, current delivery strategies for circRNAs are largely adapted from mRNA drugs, whereas specific consideration should be given to the unique structure and functions of circRNAs. To date, only a few circRNAs, such as circSCAR[17], CircRNA_0001805177 (ref. 178) and circDIDO1 (ref. 155), have used customized delivery carriers. For example, a mitochondria-targeting nanoparticle (mito-NP) has been developed to deliver circSCAR-expressing vectors to mitochondria[17]. Mito-NP is a multifunctional envelope-type LNP engineered with endosomal pH-responsive polymers to promote endosomal escape after cellular uptake[179]. Its surface is modified with triphenylphosphonium-decorated amphiphilic cationic peptides[180] to ensure specific targeting

to mitochondria. Mito-NP delivers circSCAR to the mitochondria of liver fibroblasts in high-fat-diet mice[17].

Another promising platform for circRNA delivery is metal–organic frameworks, known for their large surface area, high porosity and modifiability[181]. Encapsulating circRNA_0001805 expression plasmids into metal–organic frameworks with glycyrrhizic acid and zinc ions, and further coating them with red-blood-cell membranes, enabled immune response evasion in nonalcoholic fatty liver disease (NAFLD) mice models, as well as in hepatic tissues from patients with NAFLD[178]. Exosomes (natural extracellular vesicles carrying circRNAs[182–184]), have also been used to deliver circSCAR to attenuate sepsis in septic mice[185] and circDIDO1 to inhibit gastric cancer in xenograft mice[154]. Moreover, intra-articular injection of exosomal circEDIL3 reduced arthritis severity in a collagen-induced mouse model of arthritis[186]. As nanotechnology advances, the development of new platforms with enhanced encapsulation efficiency, stability and biodistribution will be crucial for the progress of circRNA-based therapeutics.

Notably, in vivo therapeutic efficacy of circRNAs is typically validated by overexpressing target circRNAs in diseased cell lines, followed by cell injection in xenograft tumour assays, which does not replicate the physiological environment in patients. The optimal dosage of administration also needs to be considered; unlike linear mRNAs, circRNAs have longer lifespans[19] and are more resistant to nuclease degradation[72]. Moreover, the degradation mechanism of circRNAs is not fully characterized[176]. Degradation can be mediated by Ago-2 (refs. 187,188), endonucleases[177], m^{6}A modifications[189–191], circRNA structure[192] and trimethylamine-*N*-oxide (TMAO)[193]. Hence, the dosage of circRNA therapeutics in clinical applications must take into account the various degradation pathways.

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F.Z. conceived the project. X.C. and Z.C conducted the initial literature search. X.C., J.Z. and F.Z. wrote the manuscript synopsis. All authors contributed to writing and reviewing the manuscript.

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