Circular RNAs open a new chapter in cardiovascular biology

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Abstract | Circular RNAs (circRNAs) are emerging as a new class of non-coding RNA molecules. This unusual class of RNA species is generated by a back-splicing event of one or two exons, resulting in a covalently closed circRNA molecule. Owing to their circular form, circRNAs are protected from degradation by exonucleases and have greater stability than linear RNA. Advances in computational analysis of RNA sequencing have revealed that thousands of different circRNAs are expressed in a wide range of mammalian tissues, including the cardiovascular system. Moreover, numerous circRNAs are expressed in a disease-specific manner. A great deal of progress has been made in understanding the biogenesis and function of these circRNAs. In this Review, we discuss the current understanding of circRNA biogenesis and function, with a particular emphasis on the cardiovascular system.

Circular RNAs (circRNAs) were discovered in the 1970s[1,2] but have been recognized as a novel class of non-coding RNA molecules only in the past few years. For many years, circRNAs were considered to be artefacts of RNA splicing[3], but advances in RNA-sequencing technologies have demonstrated that large numbers of circRNAs are expressed in a wide range of mammalian tissues, and interest in circRNAs has thus re-emerged[4–7]. In human tissues alone, >30,000 different circRNAs have been identified[8]. This unusual class of RNA species is generated during precursor mRNA (pre-mRNA) splicing, mostly by a back-splicing event of one or two exons. Owing to their circular form and lack of free ends, circRNAs are protected from degradation by exonucleases and have greater stability than linear RNAs[9]. Although the functions of most circRNAs are still elusive, a select number of circRNAs are known to bind microRNAs (miRNAs) to prevent their interaction with mRNA targets (that is, they act as miRNA sponges). In 2017, the first loss-of-function study in mice for a circRNA revealed that a neuron-specific circRNA regulates sensorimotor gating and synaptic transmission in the brain, thereby providing the first solid piece of evidence of a biological function of a circRNA[10].

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In this Review, we provide an overview of the current understanding of circRNA biology, with a particular emphasis on the cardiovascular system. In the first part, we discuss the general properties of circRNAs — the way they are formed and knowledge about their biological functions. In this part, we also discuss in silico and experimental approaches to study circRNAs. In the second part, we discuss the expression and function of particular circRNAs in the cardiovascular system. This part also includes a brief description of the potential use of circRNAs as biomarkers and as therapeutic carriers.

General characteristics of circRNAs

circRNAs are a heterogeneous class of transcripts. They can consist of exons only, introns only or a combination of both. Their size can range from <100 nucleotides to multiple kilobases, depending on the number of exons and/or introns located between the back-spliced exons. Mostly, they consist of 2–3 exons, with a median length of ~500–700 nucleotides[11–14]. They can be derived from protein-coding transcripts as well as non-coding transcripts, such as long non-coding RNAs. In the human brain, 20% of the genes produce circRNAs[12], whereas in the heart, ~9% of the expressed genes produce circRNAs[11]. In human leukocytes and fibroblast-like cells, 10% and 14%, respectively, of the genes produce circRNAs[13,15]. A comparison of circRNA expression in different human and mouse tissues demonstrated an overall enrichment of circRNA expression in the nervous system[12]. This relatively high expression in the nervous system might result from passive accumulation in the slowly dividing neuronal cells[16], which is in contrast to the situation in proliferative cells, in which circRNA levels are diluted when cell division takes place. In accordance with this idea, proliferating cells, including cancer cells, express lower levels of circRNAs than terminally differentiated cells[17].

Most circRNAs are expressed at 5–10% of the level of their linear transcripts[5], but dozens of circRNAs are actually expressed at much higher levels than their linear counterparts[18,19]. The generally low expression

Key points

- Circular RNAs (circRNAs) are a large class of non-coding RNA molecules that form a covalently closed loop (unlike linear RNAs).
- circRNAs are produced from precursor mRNA back-splicing, a process catalysed by the spliceosome machinery.
- High-throughput sequencing has identified thousands of circRNAs in the human body, a great number of which are expressed in a tissue-specific or disease-specific manner.
- Although the biological functions of most circRNAs remain unknown, specific circRNAs have been shown to act as microRNA sponges, to interact with RNA-binding proteins, to regulate transcription or to be translated into proteins.
- Preliminary studies have provided evidence that individual circRNAs have critical regulatory functions in the cardiovascular system.
- Owing to their high stability and abundance in bodily fluids, circRNAs have potential as useful biomarkers for various diseases, including cardiovascular disease.

level might be because back-splicing is far less efficient than canonical splicing[16]. Despite this low efficiency, their resistance to exonucleases enables some of these circRNAs to accumulate to relatively high levels.

Several studies have shown that the expression of circRNAs changes during development and disease[20]. In this regard, the expression of circRNAs has been shown to increase in developing heart, lung and brain tissues[21,22]. What causes this upregulation is unknown, as it is not explained by increased expression of their host genes. Interestingly, evidence also suggests that age-related accumulation of circRNAs occurs[23]. This accumulation might be caused by the increased stability of circRNAs, increased circRNA biogenesis or decreased proliferative capacity in ageing cells. A study using human induced pluripotent stem cell-derived cardiomyocytes showed that the expression of numerous circRNAs is dynamically regulated in these cells in conditions of chronic and acute stress (such as β-adrenergic stimulation)[24]. In addition, other stress events, such as oxidative stress or high temperatures, can also affect circRNA levels[25].

Mechanisms of circRNA formation

circRNAs are formed through a back-splicing reaction orchestrated by the spliceosome machinery[26]. This multi-protein complex normally catalyses linear pre-mRNA splicing by removing introns and joining exons together to form a mature mRNA. The formation of a circRNA requires that a donor splice site of an exon is not connected to an acceptor splice site of a downstream exon, as observed in linear splicing, but instead to an upstream acceptor site. This event creates a single or multi-exonic RNA molecule with the 5′ and 3′ ends covalently closed and that contains a unique exon–exon junction not present in the linear transcript, the so-called back-spliced junction. The mechanism by which the spliceosome selects certain exons to circularize is not fully understood but evidently requires that two introns flanking the back-spliced exons are brought into close proximity. Three mechanisms are known to achieve this step: intron pairing-driven circularization, RNA-binding protein (RBP)-driven circularization and lariat-driven circularization (FIG. 1).

Intron pairing-driven circularization

In intron pairing-driven circularization, the main components are *cis*-acting elements (short non-coding regulatory sequences) located specifically in the flanking introns of the back-spliced exons. These elements enable direct base pairing between the flanking introns and can be either short interspersed nuclear elements (SINEs; such as Alu repeats in the human genome) or non-repetitive complementary sequences[13,27–29]. The minimal intronic regions required for circRNA production have been identified[30]: miniature introns (<100 nucleotides) containing only canonical splice site sequences together with short inverted repeats are sufficient to allow exons to circularize in cells. Nevertheless, this process seems to be more complex than simple base-pairing events because not all introduced repeats lead to circularization. In this regard, the presence of mismatches in intronic repeats, low-complexity sequences (for example, poly(A) tracts) and/or secondary RNA structure might affect circularization efficiency[30]. Further studies are needed to understand how base pairing between introns might affect spliceosome assembly during back-splicing.

RBP-driven circularization

In RBP-driven circularization, *trans*-acting factors (proteins that bind *cis*-acting elements) recognize and dock on specific motifs located in the introns flanking the circularized exons. Through protein–protein interaction or dimerization of these RBPs, the splice sites are brought into close proximity and the spliceosome engages in a back-splicing reaction. The first two proteins shown to promote circRNA formation through interaction with flanking introns were protein quaking and muscleblind-like protein 1 (MBNL1)[31,32]. Other RBPs, such as RNA-binding protein 20 (RBM20), the interleukin enhancer-binding factor 3 and serine/arginine-rich splicing factors (for example, SRSF1, SRSF6 and SRSF11), have also been linked to circRNA production[33,34].

A study in *Drosophila* suggested that the two mechanisms of intron pairing-driven circularization and RBP-driven circularization might not be distinct mechanisms but might actually work together[33]. As such, intronic repeats in flanking introns are proposed to provide the opportunity for circularization to occur. circRNA formation is then further promoted by a set of proteins that function by regulating the accessibility of splice sites to the spliceosome[33]. Each gene locus might require a different set of proteins to dictate the amount of circRNAs that a cell ultimately produces.

Lariat-driven circularization

Lariat-driven circularization is the process by which circRNAs are formed during linear splicing. These circRNAs might originate during intron removal in pre-mRNA splicing[35] or from exon-containing lariats that originate during exon-skipping events[36]. Although studies have shown that circRNAs are typically not derived from alternatively spliced or skipped exons[11,15], an interesting exception is the

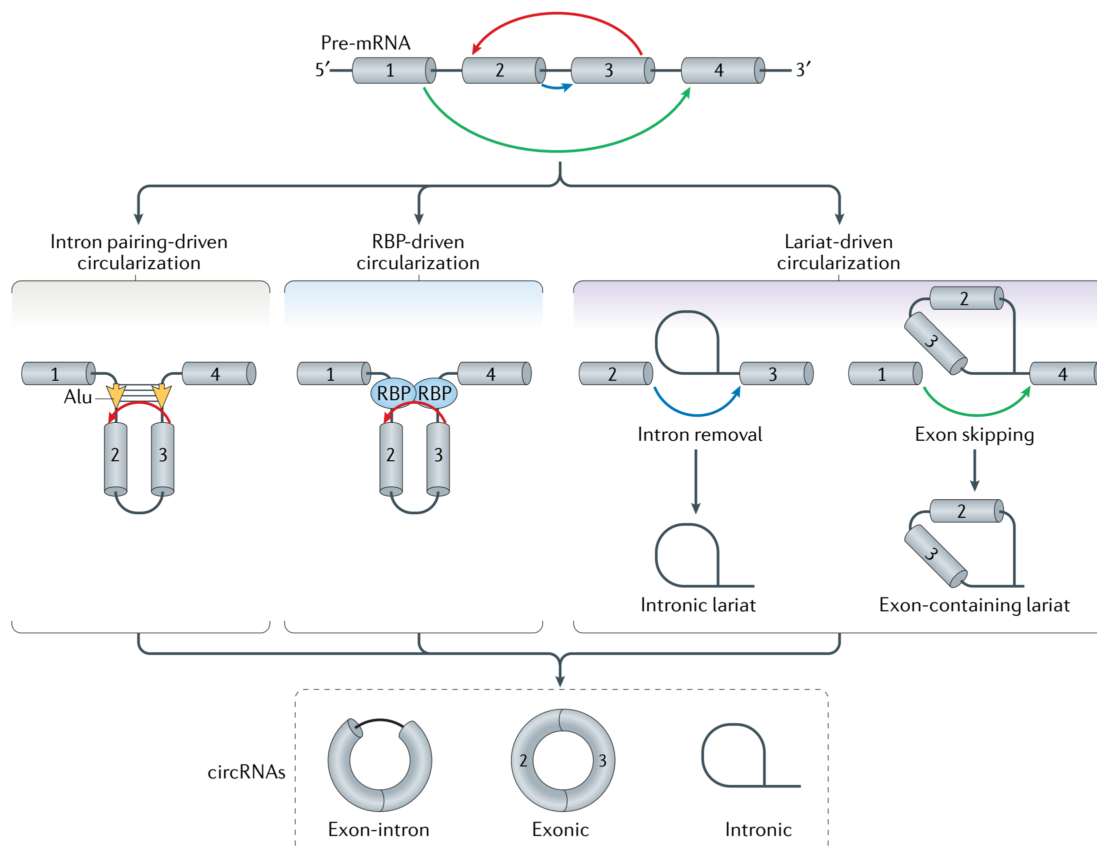

Fig. 1 | **Schematic representation showing the three mechanisms for circular RNA biogenesis.** In intron pairing-driven circularization and RNA-binding protein (RBP)-driven circularization, the back-splicing event (red arrow) is guided either by base pairing of complementary sequences located in the introns flanking the back-spliced exons or by RBPs that recognize and dock on specific motifs located in the introns flanking the circularized exons. In lariat-driven circularization, circular RNAs (circRNAs) are formed during linear splicing. These circRNAs can arise either from intronic lariats, which are formed when an intron is removed during precursor mRNA (pre-mRNA) splicing (blue arrow), or from exon-containing lariats, which originate during exon-skipping events (green arrow). In the dashed frame, the three types of circRNA that arise from the aforementioned mechanisms are shown.

TTN gene, which encodes titin. Exon-skipping events within the *TTN* transcript are an important source of circRNAs in the heart[11,37]. Why the biogenesis of *TTN* circRNA is different from that of most other circRNAs is currently unclear.

Direct base pairing of intronic elements bracketing the back-spliced exons results in double-stranded RNA. These double-stranded RNAs are prime targets for the RNA-editing enzyme ADAR (double-stranded RNA-specific adenosine deaminase), which converts adenosines into inosines by deamination. ADAR activity is proposed to destabilize the base-pairing interactions required for circRNA biogenesis. Evidence for this model came from a study demonstrating that circRNA expression correlates negatively with the expression of ADAR1 in neural tissues[12]. In accordance with this finding, introns bracketing circRNAs are highly enriched in A-to-I editing events, and knockdown of ADAR1 by small interfering RNAs (siRNAs) in a human cell line was associated with an increase in the expression of circRNAs[28]. Future studies are warranted to investigate how RNA modifications modulate circRNA biogenesis.

Methods to study circRNAs

In silico approaches

The circRNA field has grown tremendously over the past 5 years owing to advances in RNA-sequencing technologies and bioinformatics approaches. Several specialized computational pipelines or tools have been designed to identify circRNAs[38] (TABLE 1). Mostly, these prediction tools detect circRNAs by aligning RNA-sequencing reads with a reference or pseudo reference genome to subsequently extract the reads that span back-spliced junctions. However, a computational method has been developed that is independent of read-mapping to a reference genome[39]. In this approach, short sequences (called k-mers) are extracted only near the exon boundaries and reads are analysed for the presence of these out-of-order k-mers (FIG. 2). The various circRNA prediction tools differ not only in the strategy used to identify circRNAs but also in the type of circRNA they detect (that is, exonic versus intronic circRNAs). In addition, these tools have various levels of precision and sensitivity of circRNA detection and have various thresholds and criteria to define a circRNA[7,40]. Comprehensive comparisons of the performance of different circRNA

prediction tools have been reported elsewhere[38,41] and, therefore, are not discussed here. Overall, these studies conclude that no single tool outperforms the others, but that a tool should be selected on the basis of the specific needs or research questions. A combination of multiple circRNA prediction tools should ideally be used to reduce the likelihood of missing important circRNA candidates.

Experimental approaches

Irrespective of the bioinformatics tool used for circRNA prediction, validation of circRNA candidates in a given sample and proof of their circularity is required to classify them as true circRNAs. Here, we briefly discuss the most frequently used molecular assays and laboratory techniques for circRNA detection. For more detailed descriptions, see elsewhere[7,42].

Validation of circRNA expression by RT-PCR. The most widely used technique for circRNA validation is reverse transcription (RT) followed by end-point PCR or quantitative PCR (qPCR). To detect circRNAs by RT-PCR, divergent primers are designed to span the back-spliced junction (FIG. 3a). These primers specifically amplify the circRNA but not the linear mRNA. Subsequent confirmation of the presence of the back-spliced junction by Sanger sequencing in the amplicon provides a first step in the validation. On the basis of two distinct circRNA features (resistance to exonucleases and the lack of a poly(A) tail), treatment with RNase R and poly(A) RNA selection can provide further evidence of circularity[43,44] (FIG. 3b). Nevertheless, caution is needed because some validated circRNAs seem to be sensitive to RNase R, and the presence of A-stretches in circRNAs potentially obscures the poly(A) tail approach[13]. Northern blotting is another useful biochemical method to detect circRNAs. In this approach, detection relies on aberrant migration of circRNAs through a gel. By designing a probe against one or multiple exons present in the circRNA, the expression of both the linear host gene and the circRNA can be examined in a given sample. A disadvantage of northern blotting relates to the fact that lowly expressed circRNAs can be difficult to detect, as the source material is RNA, not amplified cDNA, as in RT-PCR.

Silencing and overexpression of circRNAs. To study the function of circRNAs, silencing and overexpression approaches can be used to modulate their expression in vitro and in vivo. siRNAs or short hairpin RNAs in a viral vector have been used successfully to silence circRNAs[4,25,45,46]. By designing the siRNA sequence complementary to the back-spliced junction, the RNA-induced silencing complex can be targeted to the circRNA specifically, resulting in cleavage and degradation of the circRNA (FIG. 3c). Targeting circRNAs with CRISPR–Cas9 strategies is another possibility, but a complicating factor is that simple deletion of the back-spliced exons from the genome also affects the linear transcript of the host gene. The use of CRISPR–Cas9 to disrupt circRNA expression should, therefore, be aimed at mutating and/or deleting individual intronic sequences that are required for circRNA biogenesis[14]. Most approaches rely on intron pairing-driven circularization to overexpress circRNAs. To this end, exons of a candidate circRNA are cloned in an expression vector, flanked by complementary sequences. These complementary sequences can be derived from flanking introns of efficiently generated circRNAs, such as the *laccase2* (also known as *stw*) gene in *Drosophila*, or from artificial sequences[33,47–49] (FIG. 3d).

Biological functions of circRNAs

The most pressing questions in the circRNA field are whether or not circRNAs have a biological function, and if so, what their mechanisms of action are. Currently, the most substantial piece of evidence for a biological function of a circRNA stems from mice engineered to

Table 1 | **Circular RNA detection tools**

Tool name	Website	Annotation-dependent	Strategy
CIRCexplorer2	http://yanglab.github.io/CIRCexplorer/	Yes	Fragment-based
circRNA_finder	https://github.com/orzechoj/circRNA_finder	No	Fragment-based
CIRI	https://sourceforge.net/projects/ciri/	No	Fragment-based
DCC	https://github.com/dieterich-lab/DCC	Yes	Fragment-based
find_circ	https://github.com/marvin-jens/find_circ	No	Fragment-based
KNIFE	https://github.com/lindaszabo/KNIFE	Yes	Pseudo reference-based
MapSplice	http://www.netlab.uky.edu/p/bioinfo/MapSplice	Yes	Fragment-based
NCLscan	https://github.com/TreesLab/NCLscan	Yes	Pseudo reference-based
PTESFinder	http://ibi.zju.edu.cn/bioinplant/tools/manual.htm	Yes	Pseudo reference-based and fragment-based
UROBORUS	http://uroborus.openbioinformatics.org/en/latest/	Yes	Fragment-based
segemehl	http://www.bioinf.uni-leipzig.de/Software/segemehl/	No	Fragment-based
Acfs	https://github.com/arthuryxt/acfs	No	Pseudo reference-based
CircMarker	https://github.com/lxwgcool/CircMarker	Yes	K-mer-based

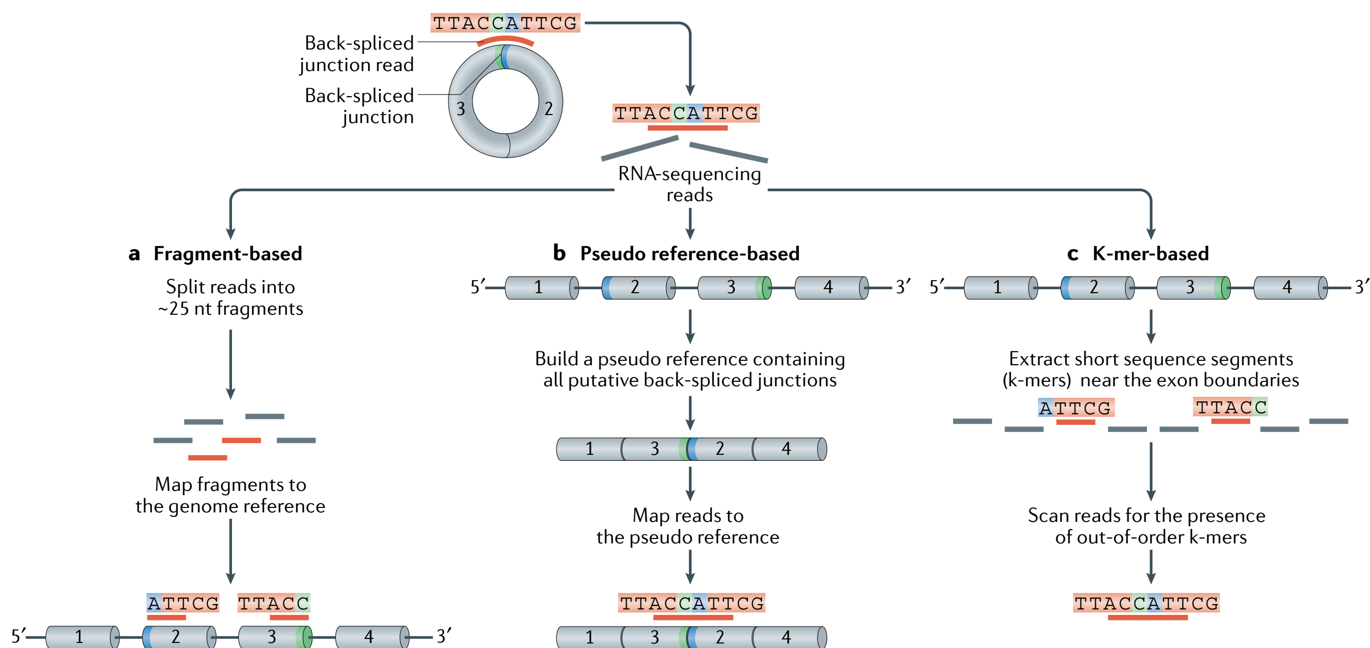

Fig. 2 | **Computational strategies for circular RNA detection. a** | In the fragment-based strategy, RNA-sequencing reads are split into fragments (~25 nt) and mapped to a reference genome. Fragments that map at back-spliced junctions are retained. **b** | In the pseudo reference-based strategy, a reference is built that contains all putative back-spliced junctions per gene (by computing all exon–exon boundary combinations). Reads that map at predicted back-spliced junctions are retained. **c** | In the k-mer-based strategy, short sequence segments (k-mers of <16 nt) are extracted near the exon boundaries (head part and tail part of the exons) of each gene. RNA-sequencing reads are then analysed for the presence of those k-mers, and reads containing out-of-order k-mers relative to the reference are retained.

lack the Cdr1as (also known as Cirs7) circRNA, which develop neurological disorders[10]. This study demonstrated that circCdr1as is required for normal synaptic transmission in the brain because this circRNA acts as a sponge for the miRNA miR-7. Nevertheless, at this stage, no general function of circRNAs has been uncovered, and several lines of evidence point towards other mechanisms of action in addition to miRNA sponging, such as sequestering proteins, regulation of gene transcription and even providing a template for protein synthesis (FIG. 4).

MicroRNA sponges

Cytoplasmic circRNAs can function as miRNA sponges by sequestering miRNAs and preventing their interactions with target mRNAs. The above-mentioned circRNA, circCDR1AS, derived from the *CDR1* locus is among the most extensively characterized circRNAs to date[4,50,51]. circCDR1AS contains a considerable number of binding sites for miR-7 (>70 in both human circCDR1AS and mouse circCdr1as[51]), and miR-7 has been shown to interact directly with circCdr1as[51]. Given that no linear *Cdr1as* transcript is detected, a knockout strategy in mice is very straightforward, and removal of the circRNA from the genome does not affect the expression of the host gene. Perturbed expression of miR-7 and increased expression of immediate early genes such as *Fos*, a direct miR-7 target in the brain, have been reported in circCdr1as-knockout mice[10]. Interestingly, loss of circCdr1as disrupted synaptic communication and induced behavioural abnormalities associated with neuropsychiatric disorders, suggesting that the interaction between circCdr1as and miR-7 is essential for normal brain function[10]. Although several other studies have reported that specific circRNAs can bind miRNAs, only a few circRNAs meet the specific stoichiometric requirement to act as an endogenous miRNA sponge[14,19,51,52]: for a sponge effect to occur, the abundance of the circRNA has to match the abundance of the miRNA. Given that most circRNAs are expressed at a low level, sponging of miRNAs does not seem to be a general function of circRNAs. This conclusion is further strengthened by the observation that bioinformatics analyses do not reveal an overall enrichment of miRNA binding sites in circRNAs[22]. Regardless, the >70 miR-7 binding sites that exist in Cdr1as make this circRNA a unique and competitive inhibitor of endogenous miR-7 targets.

Scaffolds for proteins

In addition to binding RNA molecules, circRNAs can also bind or sequester selected proteins to modulate their activity or localization. For example, a circRNA derived from the RNA transcript encoding the splicing factor MBNL1 contains multiple conserved MBNL1 binding sites, which are strongly and specifically bound by this protein[31]. The introns flanking the single back-spliced exon of *MBNL1* also contain MBNL1 binding sites, which dictate the circularization rates of bracketed exons and which depend on MBNL1 protein levels[31]. This raises the possibility of a sophisticated negative feedback system that controls MBNL1 levels. When MBNL1 protein levels are high, production of its own mRNA is decreased by promoting circMBNL1

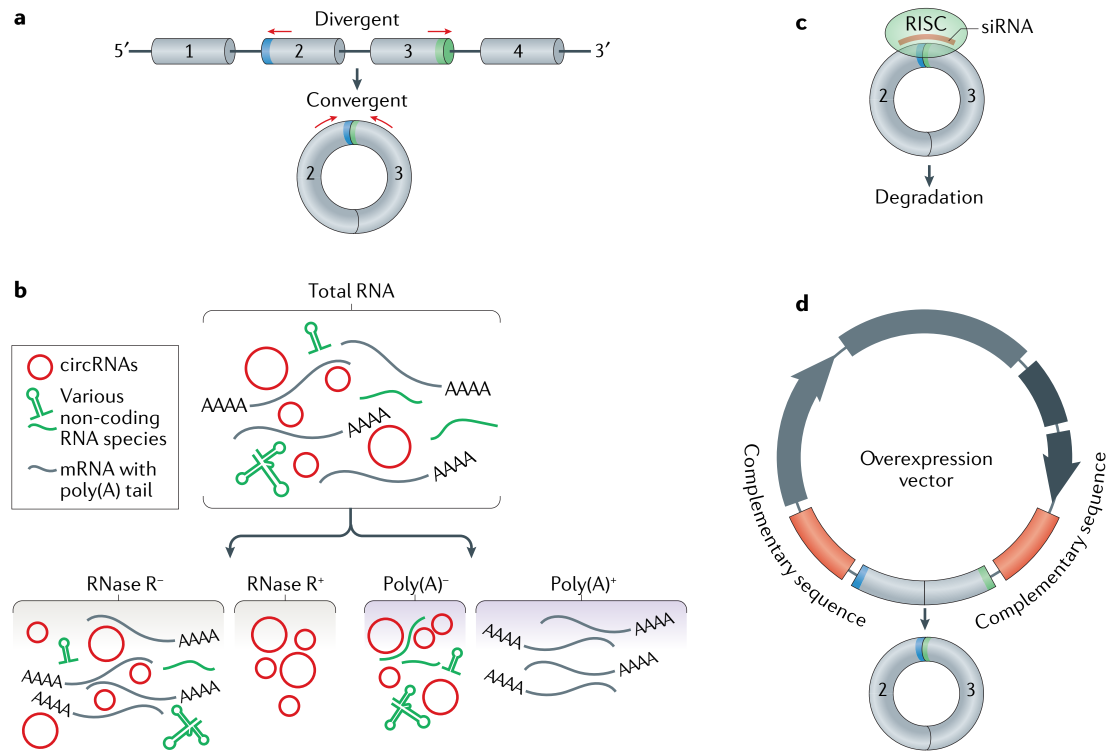

Fig. 3 | **Experimental approaches to detect and manipulate circular RNAs. a** | PCR amplification of circular RNAs (circRNAs) requires divergent primers (red arrows). These primers are divergent on the linear RNA perspective but are convergent on the circRNA. **b** | The circular nature of candidate circRNAs can be validated by treating total RNA with RNase R, which degrades linear RNAs but not circRNAs. Another feature of circRNAs is the absence of a poly(A) tail. By means of pre-selection of poly(A)$^{+}$ RNAs, linear RNAs, but not circRNAs, can be detected by PCR. **c** | To downregulate a circRNA specifically, a small interfering RNA (siRNA) directed at the back-spliced junction is used to target the RNA-induced silencing complex (RISC) to the circRNA, which triggers its degradation. Only the sequence at the back-spliced junction is specific to the circRNA and not present in the linear transcript. **d** | For overexpression of circRNAs, a plasmid is used that contains complementary sequences flanking the circRNA sequence to facilitate circularization.

production. Subsequently, this circRNA can sponge out the excess MBNL1 protein by binding to it[31].

RNA immunoprecipitation and circRNA profiling revealed that the RBP ELAV-like protein 1 (also known as human antigen R (HuR)) can potentially bind to more than half of the circRNAs present in HeLa cells[53]. One of the most prominent HuR targets is a circRNA derived from the gene *PABPN1*, and binding of HuR to circPABPN1 prevented its binding to linear *PABPN1* mRNA, thereby lowering *PABPN1* translation, which resulted in a reduction in cell proliferation[53].

A circRNA derived from the long non-coding RNA *ANRIL* (also known as *CDKN2B-AS1*), encoded on chromosome 9p21, a known hot spot for disease-associated polymorphisms (such as for cardiovascular disease and several cancers), has also been shown to affect protein translation[48,54]. circANRIL binds to pescadillo homologue, a protein of the ribosomal RNA-processing machinery, thereby impairing ribosome biogenesis and leading to activation of p53. Given that increased p53 levels seem to be atheroprotective, circANRIL has been suggested to be a potential therapeutic target for the treatment of atherosclerosis[48].

Although several proteins have been shown to interact with circRNAs, scaffolding of proteins might not be a general biological mechanism of action of circRNAs. By predicting the binding sites of 38 RBPs in circRNAs, circRNAs were revealed to have a lower RBP-binding density than either the coding sequence or the 3′ untranslated region of protein-coding genes. Therefore, on the basis of nucleotide sequence alone, circRNAs as a group are not more likely to bind to RBPs than linear mRNAs[22].

Transcriptional regulators

In addition to their role as a scaffolding molecule for miRNAs and proteins, circRNAs can also regulate transcription in the nucleus. A subclass of circRNAs that predominantly localize in the nucleus has been identified in HeLa and H9 cells[35,55]. Interestingly, this subclass of circRNAs can contain either circularized exons with the intron retained (exon–intron circRNAs) or be formed exclusively by an intronic sequence (circular intronic RNAs). The formation of circular intronic RNAs depends on a consensus motif near the 5′ splice site and close to the branchpoint site[35]. Bioinformatics analysis has shown that in 80% of circRNAs, introns are spliced out, but ~20% of circRNAs contain one or more unspliced intervening introns[19]. Biochemical and molecular characterization of some of these nuclear circRNAs (from HeLa and H9 cells) revealed that

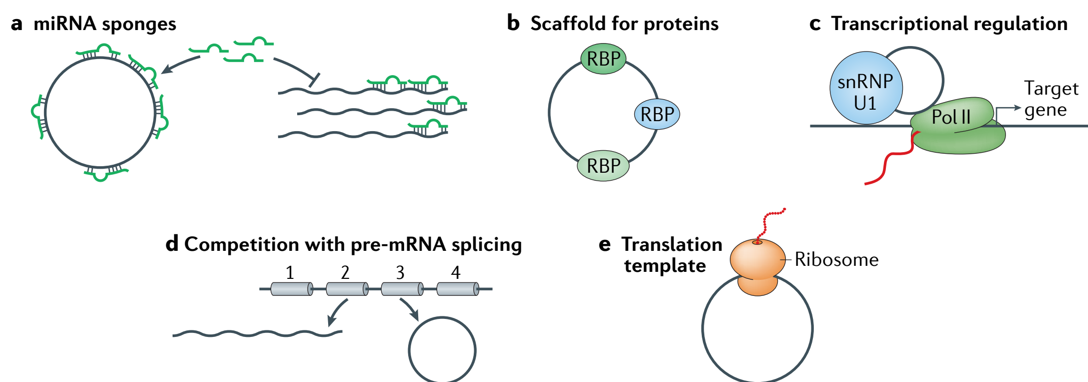

Fig. 4 | **Mechanisms of circular RNA functions. a** | Circular RNAs (circRNAs) can function as microRNA (miRNA) sponges and reduce the capacity of miRNAs to target mRNAs. **b** | circRNAs can act as scaffolds for RNA-binding proteins (RBPs) to modulate their activity or localization. **c** | circRNAs can regulate transcription by interacting with the RNA polymerase II (Pol II) machinery and small nuclear ribonucleoprotein U1 (snRNP U1) in the nucleus. **d** | circRNA biogenesis affects the expression of the host gene by competing with linear mRNA splicing. **e** | circRNAs might associate with translating ribosomes and be subjected to translation in an internal ribosome entry site (IRES)-dependent and cap-independent manner. pre-mRNA, precursor mRNA.

they regulate RNA polymerase II activity through interaction with the small nuclear ribonucleoprotein U1 (snRNP U1) and promote transcription of their parental genes[35,55].

Competitors of linear splicing

The process of circRNA biogenesis also affects the expression of the host gene. In this regard, several lines of evidence indicate that circRNA biogenesis strongly competes with linear mRNA splicing[31,56]. For instance, increasing the efficiency of linear splicing in *Drosophila* S2 cells resulted in decreased expression of circRNAs[31]. In another study, depletion of spliceosome components in *Drosophila* cells resulted in increased steady-state levels of circRNAs, whereas expression of their associated linear mRNAs concomitantly decreased[56]. As linear splicing and back-splicing mostly use the same pool of canonical splice acceptors and donors, it is not surprising that circRNAs are typically generated at the expense of linear mRNA isoforms.

Templates for protein synthesis

In addition to the non-coding functions of circRNAs, researchers have also investigated the potential of circRNAs to function as a template for translation. Given that circRNAs are mostly cytosolic and originate from protein-coding exons, the question arose as to whether they could be loaded into ribosomes and translated into proteins. Although circRNAs lack the typical 5′ cap and 3′ poly(A) tail required for translation initiation, translation might be able to proceed on a circRNA from an internal ribosome entry site (IRES). Indeed, initial findings revealed that synthetic circRNAs can be translated in vitro and in vivo when an IRES is introduced[57–59]. Ribosomal footprinting studies, however, revealed that endogenous circRNAs are not associated with ribosomes, indicating that they are generally not translated[13,22]. These findings remain controversial, because ribosome footprinting in *Drosophila* did reveal a subset of circRNAs associated with translating ribosomes[60]. Unequivocal evidence for endogenous translation from circRNAs can be obtained from mass spectrometry and the identification of circRNA-encoded peptides with unique amino acid sequences that are derived from the back-spliced junction. Two studies have provided this evidence and reported that circZNF609 in human muscle cells and circMbl in *Drosophila* heads are translated in an IRES-dependent, 5′ cap-independent manner[45,60,61]. Although translation of a circRNA does not seem to be a common function for circRNAs, an important next step in the field is to determine the physiological relevance of the few proteins that might be produced from circRNAs.

circRNA expression in the heart

In 2016, the first circRNA expression profiling study identified 575 circRNA species in mouse heart[62]. Subsequent studies performed on human heart have shown that it expresses between 7,000 and 16,000 different circRNAs[37,63,64]. Interestingly, these circRNAs seem to be poorly conserved during evolution, given that only ~5% of these human circRNAs are also expressed in mouse heart[11]. However, the rate of conservation increases (~19%) when only the highly expressed circRNAs are considered[11]. The accumulation of SINE elements, especially Alus, in the introns that flank the back-spliced exons correlates with the increased expression of circRNAs during species evolution[65].

An evolutionarily conserved single-exon circRNA from *SLC8A1* (which encodes sodium/calcium exchanger 1; NCX1) is the most abundantly expressed circRNA in the human heart[37,63,64]. circSLC8A1 is expressed at a level 40 times higher than that of the linear *SLC8A1* mRNA[37,63,64]. In 1999, this particular circRNA was identified on a northern blot when a small, highly abundant 1.8 kb RNA transcript was noted in addition to the full-length (13 kb) transcript of *SLC8A1* from heart and various other tissues[66]. This 1.8 kb RNA band corresponded to a perfectly circularized exon 2, containing the normal *SLC8A1* start codon and a newly formed stop codon that was introduced as a result of

the circularization. The open reading frame within circSLC8A1 encodes a 602-amino-acid NCX1 protein. Experimental overexpression of a linear version of the circSLC8A1 revealed a protein matching the predicted size, but this protein could not be detected in native tissue[66]. Therefore, further research is needed to provide evidence for the endogenous expression of a 60-kDa truncated NCX1 protein to prove that this protein is derived from protein synthesis of the circular transcript and to elucidate the function of circSLC8A1 and its presumed protein product.

TTN and *RYR2* produce the highest number of different circRNAs in the human heart, with each expressing >50 different circRNA isoforms[37,63]. Although *TTN* is consistently reported to produce by far the most circRNA isoforms in the human heart, owing to differences in sequencing depth and filtering criteria in circRNA detection algorithms, the exact number of expressed *TTN* circRNAs varies substantially between studies. Work from our group has shown a total of 80 circRNAs to arise from *TTN*, whereas others have detected >400 circRNAs to arise from *TTN* in the human heart[37,64]. Most of the TTN circRNA isoforms are derived from a specific region within the gene, the so-called I-band, which is known to undergo extensive alternative splicing. The inclusion of this I-band, which forms a molecular spring in the titin protein, determines the passive stiffness of the sarcomere and thereby ultimately the passive stiffness (or elasticity) of the heart[67]. RBM20 is the splicing factor responsible for alternative splicing within this I-band region, and pathogenic variants in *RBM20* result in the expression of large and compliant titin isoforms, a process believed to be an important reason why carriers of *RBM20* mutations universally develop dilated cardiomyopathy (DCM)[68–70]. RBM20 is also crucial in the biogenesis of a specific subset of TTN circRNAs in the heart[37]. We are currently studying the biological function of some of these TTN circRNAs to address whether mutations in *RBM20* might induce myocardial disease not only by abnormal splicing of linear transcripts but also through their capacity to control circRNA production.

Protein quaking is yet another splicing factor that has been linked to the biogenesis of circRNAs. Initially, protein quaking was reported to regulate circRNA formation during epithelial–mesenchymal transition[32]. A 2018 study provided evidence that protein quaking also regulates the expression of certain circRNAs in the mouse heart, in particular, circRNAs derived from *Ttn*, *Fhod3* and *Strn*[71].

circRNA expression in diseased hearts

A circRNA expression profiling study in three hearts from non-diseased control individuals, three hearts from patients with non-ischaemic end-stage heart failure (that is, DCM), three hearts from patients with ischaemic cardiomyopathy (ICM) and three hearts from patients with hypertrophic cardiomyopathy (HCM) did not reveal significant differences in circRNA expression between the four groups[64]. Similarly, RNA sequencing in 12 control versus 13 hypertrophic mouse hearts (3 weeks of thoracic aorta banding) did not reveal significant differences in circRNA expression[64]. In a second study, in which two control, two HCM and two DCM human hearts were analysed for circRNA expression, 103 potential disease-regulated circRNAs were identified[37]. Validation by RT-PCR in tissues from a larger group of individuals (seven healthy controls, seven with HCM and seven with DCM) revealed that specific circRNAs arising from *CAMK2D* and *TTN* were downregulated in HCM and DCM, respectively[37]. Expression of the host genes was not altered, indicating that the disease-regulated changes in circRNA production are independent of transcriptional regulation[37]. In a third circRNA profiling study, a tendency towards increased levels of circRNA expression in human failing hearts compared with non-failing hearts was observed[63]. In a fourth study, the Arraystar Human circRNA Array was used to analyse circRNA expression in the diabetic mouse myocardium[72]. Considering the low evolutionary conservation of circRNAs, the use of a human circRNA array to detect mouse circRNAs does not seem to be a robust approach. Nevertheless, 76 differentially expressed (greater than twofold) circRNAs were identified in the myocardium of the diabetic mice compared with the myocardium of control mice, and the researchers confirmed the dysregulation of at least one circRNA (derived from *Myo9a*) by RT-qPCR in the diabetic mouse heart. In conclusion, these initial profiling studies in diseased hearts show incongruent results, but some studies do reveal various differentially expressed circRNAs in heart disease. A serious limitation of these studies is their small sample size. Moreover, the differences in methodologies used (computational or wet laboratory) make them difficult to compare. Further studies with large cohorts are needed to examine the expression changes in heart disease in greater detail.

circRNAs in the cardiovascular system

To date, only a few studies have demonstrated a biological function of a circRNA in the cardiovascular system (TABLES 2,3). The studies with the strongest available evidence for a biological function are discussed in more detail here. The first study to reveal a biological function for a circRNA in the heart was published in 2016 (REF.[47]). This study demonstrated that a circRNA, derived from *PWWP2A*, acts as an endogenous miR-223 sponge to inhibit cardiac hypertrophy and heart failure. This heart-related circRNA (named HRCR) contains six predicted miR-223 binding sites, and pull-down assays demonstrated that miR-223 binds directly to HRCR. Moreover, knockdown of HRCR in cultured cardiomyocytes resulted in reduced levels of endogenous target genes of miR-223, demonstrating that this circRNA competes with endogenous miRNA targets for miR-223 binding. Intravenous adenoviral delivery of HRCR attenuated cardiac hypertrophy and improved cardiac function after isoprenaline treatment in mice. Given that the expression level of HRCR was reduced in the mouse failing hearts, normalization of HRCR levels has been suggested as a therapeutic target for the treatment of heart failure[47].

A circRNA derived from *Foxo3* (which encodes a member of the forkhead family of transcription factors)

Table 2 | **Circular RNAs with a function in the heart**

Circular RNA	Host gene	circBase ID[a]	Function	Mechanism	Targets	Refs
In vivo studies						
HRCR	*PWWP2A*	• hsa_circ_0074837 • mmu_circ_0000254	Overexpression attenuates cardiac hypertrophy	miRNA sponge	miR-223	47
circFoxo3	*FOXO3*	• hsa_circ_0006404 • mmu_circ_0002207	Promotes cardiac senescence and aggravates doxorubicin-induced cardiomyopathy	Protein binding	ID1, E2F1, FAK and HIF1α	25
MFACR	*SMYD4*	• hsa_circ_0004018 • mmu_circ_0000290	Mediates cardiomyocyte apoptosis and ischaemia–reperfusion-induced cardiac dysfunction	miRNA sponge	miR-625-3p	82
circAmotl1	*AMOTL1*	• hsa_circ_0004214 • Mmu not in circBase	Reduces cardiomyocyte apoptosis and protects against doxorubicin-induced cardiomyopathy	Protein binding	AKT and PDK1	73
Cdr1as	*CDR1*	• hsa_circ_0001946 • mmu_circ_0001878	Controls excitatory synaptic transmission; overexpression promotes apoptosis and aggravates infarct injury	miRNA sponge	miR-7 and miR-671	10,83
In vitro studies						
circRNA_000203	*MYO9A*	• hsa_circ_0036167 • mmu_circ_0001784	Stimulates profibrotic gene expression in cardiac fibroblasts	miRNA sponge	miR-26b-5p	72
circZNF609	*ZNF609*	• hsa_circ_0000615 • mmu_circ_0001797	Controls myoblast proliferation	Protein coding and miRNA sponge	miR-194-5p	45,84
circRNA_010567	*ZSWIM6*	• hsa not in circBase • mmu_circ_0000498	Required for fibrotic gene expression in cardiac fibroblasts	miRNA sponge	miR-141	85

hsa, human; miRNA, microRNA; mmu, mouse. [a]circBase is a public circular RNA database[8] with merged and unified data sets from multiple studies across various cell types and tissues; circular RNAs in circBase are given a unique identifier (that is, a circBase ID).

is expressed at higher levels in aged hearts than in young hearts in both humans and mice[25]. The expression of this circRNA (circFoxo3) correlated with markers of senescence, and ectopic expression of circFoxo3 promoted cellular senescence in cultured fibroblasts. Moreover, doxorubicin-induced cardiomyopathy was aggravated by in vivo overexpression of circFoxo3 and was relieved after silencing of endogenous circFoxo3 (REF.[25]). circFoxo3 was found to interact with DNA-binding protein inhibitor ID1, transcription factor E2F1, focal adhesion kinase 1 and hypoxia-inducible factor 1α, leading to the relocation of these senescence-related proteins to the cytoplasm and depleting them from the nucleus[25]. The same research group reported on the function of another circRNA that is derived from the *Amotl1* gene[73]. Whereas in vitro overexpression of circAmotl1 improved cardiomyocyte survival and decreased apoptosis, silencing of circAmotl1 led to decreased survival and increased levels of apoptosis. In a mouse model of doxorubicin-induced cardiomyopathy, circAmotl1 overexpression was found to be cardioprotective, as evidenced by reduced left ventricular dilatation, less collagen deposition and a decreased number of apoptotic cells[73]. Mechanistically, the authors revealed that circAmotl1 facilitates the activation of AKT — a central node in cardiomyocyte signalling — and its subsequent nuclear translocation[73].

A circRNA derived from the *Myo9A* transcript (that is, circRNA_000203) was found to be increasingly expressed in diabetic mouse hearts compared with healthy mouse hearts[72]. Functionally, circRNA_000203 increased the expression of *Col1a2*, *Col1a3* and *Acta2* in cultured cardiac fibroblasts by sponging miR-26b-5p and derepressing its downstream targets *Ctgf* and *Col1a2*. This circRNA might be an interesting target for the treatment of cardiac fibrosis[72].

A circRNA derived from the *ZNF292* gene was found to be regulated in response to hypoxia in endothelial cells[46]. This circRNA had proangiogenic activities in vitro, given that silencing of circZNF292 reduced tube formation and sprouting of endothelial cells. Further studies are needed to find a putative proangiogenic role of circZNF292 in the heart.

circRNAs as blood biomarkers

The stability of circRNAs makes them interesting candidates as disease biomarkers in blood. A strikingly high number (that is, >2,400) of different circRNAs have been reproducibly detected in human whole blood[74]. In addition, various studies have identified ~1,000 circRNAs in

exosomes from human serum[75], a total of 19 circRNAs in human plasma during pregnancy[76] and >400 circRNAs in saliva[77]. Intriguingly, the expression profile of serum-derived exosomal circRNAs could be used to distinguish patients with colon cancer from healthy control individuals[75]. A few circRNAs have also been implicated as potential blood biomarkers in cardiovascular disease. For instance, carriers of the coronary artery disease-protective haplotype (9p21) showed markedly increased expression of circANRIL in peripheral blood mononuclear cells and in whole blood compared with non-carriers[48]. The expression level of myocardial infarction-associated circRNA (MICRA), measured at reperfusion in blood samples from patients with acute myocardial infarction, predicted the presence of left ventricular dysfunction (defined as an ejection fraction ≤40%) after 4 months[78]. A circRNA, hsa_circRNA_025016, derived from the transcript of the α1-subunit of the L-type calcium channel (encoded by *CACNA1C*), has been shown to have potential as a plasma biomarker for the prediction of postoperative atrial fibrillation[79].

Conclusions

Interest in circRNAs is growing, and work from the past 5 years has provided evidence that at least some individual circRNAs have biologically relevant functions. However, the function of thousands of circRNAs remains elusive, and whether the majority of these circRNAs have a biological role or whether they merely represent transcriptional noise remains unknown. Conclusive in vivo studies uncovering circRNA function are rather limited because specific interference in circRNA expression is technically challenging. In this regard, splicing and circRNA production are intertwined, and targeting of a circRNA using CRISPR–Cas9 strategies is likely to affect splicing or expression of the linear host gene. In addition, designing siRNAs against circRNAs is not easy because these siRNAs need to be designed precisely against the ~20 nucleotides that flank the back-splice junction to avoid binding of the siRNA to the linear mRNA. Despite these known drawbacks, the expression of some circRNAs can be specifically knocked down using siRNAs or short hairpin

Table 3 | **Circular RNAs with a function in the vascular system**

Circular RNA	Host gene	circBase ID[a]	Function	Mechanism	Targets	Refs
In vivo studies						
circHIPK3	*HIPK3*	• hsa_circ_0000284 • mmu_circ_0001052	Required for diabetic retinopathy	miRNA sponge	miR-30a-3p	86
circZNF609	*ZNF609*	• hsa_circ_0000615 • mmu_circ_0001797	Required for diabetic retinopathy	miRNA sponge	miR-615-5p	87
circSHKBP1	*SHKBP1*	• hsa_circ_0000936 • mmu not in circBase	Required for glioma angiogenesis	miRNA sponge	miR-544a and miR-379	88
In vitro studies						
cZNF292	*ZNF292*	• hsa_circ_0004383 • mmu not in circBase	Required for angiogenic sprouting in HUVECs	Unknown	Unknown	46
circANRIL	*ANRIL*	• hsa_circ_0008574 • mmu not in circBase	Induces apoptosis and inhibits proliferation in SMCs	Protein binding	Pescadillo homologue	48
circACTA2	*ACTA2*	• hsa not in circBase • mmu not in circBase	Stimulates *ACTA2* expression and regulates SMC contraction	miRNA sponge	miR-548f-5p	89
hsa_circ_0010729	*HSPG2*	• hsa_circ_0010729 • mmu not in circBase	Regulates proliferation and apoptosis in hypoxic HUVECs	miRNA sponge	miR-186	90
hsa_circ_0003575	*CHMP5*	• hsa_circ_0003575 • mmu not in circBase	Inhibits proliferation and angiogenesis capacity in HUVECs	Unknown	Unknown	91
circWDR77	*WDR77*	• hsa_circ_0013509 • mmu not in circBase	Regulates VSMC proliferation and migration	miRNA sponge	miR-124	92
circ_0005015	*HAS2*	• hsa_circ_0005015 • mmu not in circBase	Regulates proliferation and migration in retinal endothelial cells	miRNA sponge	miR-519d-3p	93
hsa_circ_0054633	*PNPT1*	• hsa_circ_0054633 • mmu not in circBase	Protective in glucose-induced dysfunction in HUVECs	miRNA sponge	miR-218	94

HUVEC, human umbilical vein endothelial cell; miRNA, microRNA; SMC, smooth muscle cell; VSMC, vascular smooth muscle cell. [a]circBase is a public circular RNA database[8] with merged and unified data sets from multiple studies across various cell types and tissues; circular RNAs in circBase are given a unique identifier (that is, a circBase ID).

RNAs[4,25,45,46]. We envisage that a better understanding of circRNA biogenesis might enable us to mutate individual intronic sequences using genome-editing tools to disrupt the expression of a specific circRNA in vivo, without interfering in the expression or splicing of the host gene. Despite these limitations, circRNAs are emerging as potentially important regulators of cellular physiology and add another level of regulation to the field of non-coding RNAs.

The identification of large numbers of circRNAs in bodily fluids holds promise that circRNAs might be used as biomarkers for various diseases. However, this research is still in its infancy, and techniques and bioinformatics methods for the reliable detection of circRNAs need standardizing. In this respect, the general comparison to miRNA biomarker research is evident. In the past decade, a great number of studies have identified circulating miRNAs with biomarker potential; however, the large number of studies with incomparable designs and methods has resulted in very low replicability of these studies so that miRNA-based biomarkers have not yet been translated towards clinical care. Nevertheless, guided by the remarkably rich circular transcriptome in whole blood, scientists will undoubtedly be encouraged to embark on studies in the coming years to seek biomarkers for diagnosis, prognosis and therapeutic guidance in cardiovascular disease. Given that circRNAs are contained in extracellular vesicles, an interesting strategy might be to capture tissue-specific and disease-specific vesicles to measure circRNA profiles therein[80]. These profiles might better predict disease onset, severity and progression than measurements in whole blood.

Future developments in the circRNA field might also relate to their therapeutic potential. The high stability of circRNAs makes them potential long-lasting regulators of specific cellular functions. For instance, engineered circRNA overexpression constructs could be tailored for miRNA binding, RBP binding or even to overexpress proteins by including IRES sequences. According to a 2018 study, overexpression of an artificial circRNA was able to sponge the liver-specific miRNA-122, which is required for the life cycle of the hepatitis C virus (HCV)[81]. The engineered circRNA sponge (containing eight seed-matched miR-122 binding sites) inhibited viral protein production in an HCV cell culture system. The inhibitory efficiency was significant compared with the control condition and similar to the effect of the locked nucleic acid oligonucleotide drug miravirsen, which targets the virus by complementary binding to miR-122 (REF.[81]). Taken together, artificial circRNA constructs with a wide range of potential applications are a promising tool in molecular medicine.

In conclusion, we have discussed the evidence that specific circRNAs have biologically relevant functions. New information and insight into the biogenesis and function of circRNAs are being generated rapidly; however, understanding the contribution of circRNAs to the development and progression of cardiovascular disease is still in its infancy. The next few years will be an exciting period for the circRNA field when more functions and disease mechanisms will be uncovered.

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Author contributions

S.A. and E.E.C. contributed to researching data for the article, discussion of content, writing, reviewing and editing the manuscript before submission. Y.J.R. contributed to researching data for the article, writing, reviewing and editing the manuscript before submission. Y.M.P. contributed to reviewing and editing the manuscript before submission.

Competing interests

The authors declare no competing interests.

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