Perspective

Circular RNAs and competing endogenous RNA (ceRNA) networks

Apratim Mitra, Karl Pfeifer, Ki-Sun Park

Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health, and Human Development, National Institutes of Health, Bethesda, MD, USA

Correspondence to: Ki-Sun Park. Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 31 Center Dr., Bldg 6B-Room 2B206, Bethesda, MD, USA. Email: ki-sun.park@nih.gov.

Abstract: In recent years, advances in bioinformatics approaches have allowed a systematic characterization of circular RNAs (circRNAs) across a variety of cell types. Demonstration of cell type specificity, regulated expression, and conservation between species all suggest that circRNAs have functional importance. Especially, investigators have begun focusing on the possibility that circRNAs operate as part of competing endogenous RNA (ceRNA) regulatory networks that are proposed to play critical roles in normal development and in pathologic conditions like cancer.

Keywords: Circular RNAs (circRNAs); microRNAs; miRNA; ceRNA networks

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What is an exonic circRNA?

Exonic circular RNAs (circRNAs) are covalently closed, single-stranded molecules produced by backsplicing of pre-mRNA transcripts (*Figure 1*) (1-3). Backsplicing is accomplished by the same splicesome machinery that generates mRNAs and exonic circRNAs carry 3',5'-phosphodiester bonds at their backsplice junctions. However, during circRNA synthesis, splicing does not follow the canonical 5'-3' order. Like linear mRNAs, exonic circRNAs are transported to the cytoplasm. Consistent with their circular nature, circRNAs lack the terminal structures [5' caps and poly(A) tails] associated with their linear counterparts and are resistant to exonucleases like RNAse R. Possibly because of their resistance to exonuclease digestion, circRNAs are generally more stable *in vivo* than linear transcripts.

Intronic circular RNAs also exist. These are also formed by the normal spliceosome machinery but are biochemically distinct: they contain a 2'-5' linkage at their backsplice junctions. Intronic circRNAs remain nuclear and accordingly are proposed to have distinct functions from exonic circRNAs. This Perspective will focus specifically on exonic circRNAs.

Recent advances in genomic analyses have enabled systemic study of exonic circRNAs

circRNAs were first identified at least by 1991 (4) and in subsequent years several other circRNAs were fortuitously identified. However, major advances in the field occurred only recently upon application of novel and clever innovations to genome-wide analyses of RNA. In regard to sample prep, key technology advances include the use of ribosome depletion as an alternative to poly(A) purification for enrichment of mRNA and the use of RNAse R treatment to enrich for circular RNAs. Despite these enrichment steps, circRNAs can be difficult to identify/map and additional procedures are necessary to improve sensitivity. First, due to their low abundance, longer reads (100 bp or more) and increased depth of sequencing are essential to obtain sufficient sequence around the backsplice junctions. Second, sequencing samples before and after RNAse R treatment can help distinguish linear mRNA from circRNAs. Finally, novel computational pipelines take advantage of the non-canonical 3'-5' orientation of circRNA splicing. Combining these approaches, multiple labs have now characterized circRNAs across multiple cells types and species, and within various developmental

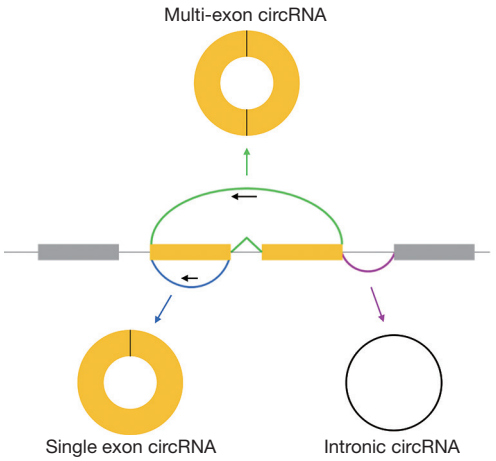

Figure 1 Schematic showing different classes of circRNAs. Filled rectangles represent exons of a spliced transcript and solid straight lines represent introns. Colored arcs represent different types of backsplicing events: (I) the blue arc corresponds to single-exon backsplicing leading to a single exon circRNA; (II) the green arc represents multi-exon backsplicing which produces a multi-exon circRNA. In this case, the angled green lines represent canonical splicing between back-spliced exons; (III) the pink arc represents lariat intron backsplicing resulting in an intronic circRNA.

and disease states. One very recent example of this type of analysis is described by Chen and colleagues who compared circRNA in fetal and adult muscle cells (5).

Several websites have been developed to catalog data collected from genomic analyses conducted across multiple labs including circBase (www.circbase.org), circ2Traits (http://gyanxet-beta.com/circdb/), circInteractome (https://circinteractome.nia.nih.gov/), circRNAdb (http://reprod.njmu.edu.cn/circrnadb), and PlantcircBase (http://ibi.zju.edu.cn/plantcircbase/).

General features of exonic circRNAs

circRNAs have been identified in plants, fungi, and in all metazoans examined, including *C. elegans*, *Drosophila melanogaster*, mice, and humans. In human fibroblasts, at least 10% of all transcribed genes generate circRNA isoforms (6). Across all cell types, it is likely that 50% of all genes have a circRNA isoform. Therefore, circRNAs are not oddities observed only at rare genes.

Several analyses suggest that in human cells, exonic circRNAs are present at about 1% of the levels of poly(A) RNA (3). These low levels at first suggested that circRNAs were unlikely to be functionally significant and that they existed only as a consequence of "errors" in the splicing process. However, more recent findings have provided evidence to suggest that circRNAs are functional. First, dozens of loci have been identified where circRNAs are more abundant than the linear isoforms (7). Second, many circRNAs are expressed in a cell type-, tissue-, developmental-, aging-, or disease-specific manner that does not always correlate with changes in the expression of the linear isoform (3). Third, conservation of circRNAs across species has been demonstrated (3). Fourth, and perhaps most interestingly, specific mechanisms for regulating expression of circRNA have been identified at some loci (8,9), demonstrating that at least in regards to these genes, evolution has acted to control how much circRNA is available in the cell.

Technically, it is now established that circRNA levels can be regulated by siRNA. This is important because it has allowed investigators to target specific circRNAs to directly test their role in normal cell function. *Table 1* lists just a few examples of the many recent studies supporting functionality for specific circRNAs.

How can circRNAs affect cell function?

CircRNAs and peptide synthesis

About 40% of circRNAs carry AUG start codons that could in principle initiate translation of short peptides. In addition, investigators have demonstrated that engineered circular RNAs introduced into the cell can interact with ribosomes to generate protein (3). Moreover, at least one naturally occurring circRNA has been shown to direct peptide synthesis (19). However, in general, circRNAs are not associated with ribosomes and for now, peptide synthesis is not considered a likely role for most circRNAs. Instead, analysis of circRNA function has focused on their ability to interact with other RNAs to influence gene expression.

CircRNAs and competing endogenous RNA (ceRNA) networks

A profound breakthrough of recent genomic analyses has been the discovery that a significant proportion of the mammalian transcriptome does not correspond to annotated exons of protein-coding genes. Instead much of the transcriptional machinery is used to generate transcripts

Table 1 circRNAs and disease

Disease	Characterization	circRNA	Expression level	Proposed biological function	Citation
Cancer	Breast cancer	circ-Foxo3	Downregulated	circ-Foxo3-p21-CDK2 axis arrested the function of CDK2 and inhibited cell cycle progression	(10)
	Gastric cancer	circPVT1	Upregulated	circpVT1 promotes cell proliferation through miR-125 interactions	(11)
	Liver cancer	circHIPK3	Upregulated	circHIPK activates cell proliferation by sponging miR-124	(12)
	CRC	hsa_ circ_001569	Upregulated	hsa_circ_001569 promotes cell proliferation and invasion by sponging miR-145	(13)
Neurological disease	Alzheimer's disease	CDR1as	Downregulated	Deficiency of CDR1as contributes to AD through upregulation of miR-7	(14)
Diabetes	Diabetic retinas	circHIPK3	Upregulated	circHIPK3 may contribute to elevated levels of VEGFC, FZD4, and WNT2 expression by blocking miR-30a-3p	(15)
Heart disease	Myocardial infarction	CDR1as	Upregulated	Cdr1as functions as a miR-7a sponge in myocardial cells	(16)
	Hypertrophic and failing hearts	HRCR	Downregulated	HRCR protects against pathological hypertrophy by targeting miR-223	(17)
Aging	Cellular senescence	circPVT1	Upregulated	circPVT1 suppresses cellular senescence by inhibiting miRNA let-7	(18)
Skeletal muscle disease	Duchenne muscular dystrophy	circZNF609	N/A	circZNF609 controls myoblast proliferation and encodes a protein	(19)
	Myogenesis	circLMO7	N/A	circLMO7 regulates myoblast differentiation and survival by sponging mir-378-3p	(5)

circRNA, circular RNAs.

from pseudo-genes, long non-coding RNAs, and exonic circRNAs that do not encode peptides. Recent theoretical and experimental studies suggest that all these non-coding RNAs act to regulate each other and to regulate levels of coding transcripts by interacting with a limited pool of microRNAs (miRNAs) (*Figure 2*) (20,21).

miRNAs are 22-nucleotide long species, processed from longer precursor mRNAs, that bind to 5–7 bases of complementary sequences on target RNAs to destabilize the target RNA and/or to reduce translation frequency (22,23). Thus miRNAs efficiently downregulate gene function. There are now thousands of miRNAs identified in mammalian cells. Target genes can have multiple miRNA binding sites (called MREs for miRNA response elements) and it is estimated that miRNAs thereby can regulate a large proportion of the transcriptome. In fact, miRNAs have been implicated in important developmental pathways, stress responses, and in numerous diseases including cancer.

circRNAs (like lncRNAs or pseudogenes) also carry MREs and thus have the ability to modulate miRNA activity by binding miRNA molecules and thereby reducing their availability to bind to and downregulate protein coding transcripts. This activity is often referred to as a "molecular sponge effect" or "sponging". Because miRNAs invariably target multiple coding RNAs, a molecular sponge will affect expression of multiple genes. Thus circRNAs, along with their lincRNA and pseudogene accomplices, could form a large-scale regulatory network across the transcriptome.

Analysis of several model systems provides experimental support for the hypothesis that competition for miRNA regulates important biological functions. Many circRNAs described in *Table 1* are proposed to work via titration of miRNAs. For example, Wei *et al.* indicate that circLMO7 regulates myoblast differentiation and survival by sponging mir-378-3p (5).

Despite this experimental support, there remain reasons for skepticism regarding the ability of many circRNAs to function as miRNA molecular sponges (24,25). Denzler *et al.* performed a very careful analysis of the ability of molecular sponge molecules to interfere with miR-122 targets and saw that sponging was observed only after the addition of $>10^5$ MRE sites per cell (24). These researchers suggest that

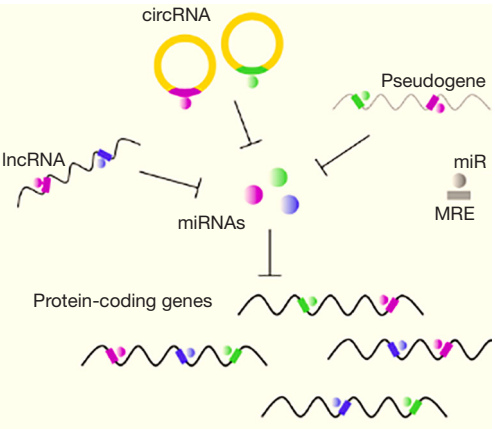

Figure 2 Network of competing endogenous RNAs (ceRNAs) and microRNAs. MicroRNAs repress protein coding mRNAs by binding to miRNA response elements (MREs) that are 7–9 bp in length and abundant in the transcriptome. Each miRNA can regulate multiple mRNAs and most mRNAs are regulated by more than one miRNA. Different classes of ceRNAs, namely circRNAs, lncRNAs and pseudogenes, also contain MREs and compete for miRNA binding, thereby regulating miRNA repression. Thus, mRNAs, ceRNAs, and miRNAs exist in a network of interactions. The existence of this network has two important implications. First, the density of the network connections means that only a very large change in the amount of any one ceRNA species can significantly impact downstream mRNA targets. Second, any such ceRNA effect would likely not be restricted to only a few loci but would affect all mRNAs carrying the same MRE. To date most studies on circular RNA function have focused on a few specific mRNA targets but the ceRNA hypothesis suggests that genome-wide analyses are necessary to fully assess the function of these molecular sponges.

MRE frequency per cell is already so high (partly because MREs are only 5–7 nucleotides and therefore present on many molecules) that expression of any single endogenous competitor would have to be extraordinarily high to be an effective sponge. As an alternative mechanism, it is interesting to consider that molecular sponges might work by regulating availability of RNA binding proteins (RBPs). RBPs might be more specific, in that they have fewer targets per cell than miRNAs, and therefore may be more susceptible to competition.

Summary

Advances in technologies for RNA deep sequencing and the development of specialized pipelines are allowing researchers to better characterize circRNA expression patterns. Some initial experiments support the idea that these molecules are more than random splicing errors and can have important biological functions. Currently much research is focused on circRNAs being part of a ceRNA network but continued work is required to determine just how important this process is to normal cell functioning.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.05.12). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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