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Molecular functions and specific roles of circRNAs in the cardiovascular system

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ABSTRACT

As part of the superfamily of long noncoding RNAs, circular RNAs (circRNAs) are emerging as a new type of regulatory molecules that partake in gene expression control. Here, we review the current knowledge about circRNAs in cardiovascular disease. CircRNAs are not only associated with different types of cardiovascular disease, but they have also been identified as intracellular effector molecules for pathophysiological changes in cardiovascular tissues, and as cardiovascular biomarkers. This evidence is put in the context of the current understanding of general circRNA biogenesis and of known interactions of circRNAs with DNA, RNA, and proteins.

1. Introduction

Today, nearly 28000 genes for long noncoding RNAs (lncRNAs) have been mapped. These lncRNA genes can be located in genomes separate from any other protein-coding gene, or overlapping in complex patterns with other genes [1]. LncRNAs have been shown to be expressed in a cell type- and state-specific mode during cell differentiation and during pathophysiological cell state changes including in diseases of the cardiovascular system [2]. Surprisingly, although genes are linear, and mRNAs are linear, and although typical lncRNA transcripts are linear as well, cells also express circular non-protein-coding RNAs, termed “circRNAs”. In a seminal study by Salzman et al. [3], advances in high-throughput sequencing and in annotating splice-junctions have allowed accumulating solid evidence that thousands of eukaryotic genes form circular RNAs as a physiologically normal process (see Ref. [4] for review). These circRNAs are not encoded as separate genetic units, like lncRNAs, but are produced by a variant form of splicing from the pre-mRNA of any transcribed gene *in cis*, may it be protein-coding or noncoding. To form circRNAs, the spliceosome covalently fuses an RNA's 5′ end to its 3′ end, which results in a circular ribonucleic acid. Thus, circRNAs lack 5′Cap and poly(A) tail. These features are used to biochemically enrich for circRNAs. For example, one can negatively select against polyA-containing mRNA, or digest all linear RNAs in circRNA preparations by adding exonucleases like RNase R that attack open linear ends [5]. Irrespective of origin, and with only a tiny minority of exceptions, circRNAs are not translated into proteins and are thus falling into the class of noncoding RNAs. More specifically, with a median size of around 500 ribonucleotides, circRNAs are part of the larger superfamily of lncRNAs [3,6–9].

Two seminal findings showed that circRNAs do have important regulatory and developmental functions in animals [8,10]. This triggered a number of studies on circRNAs in basic and applied research, including studies relevant to cardiovascular research. Despite the large number of annotated circRNAs, so far only a handful of these has been approached by functional studies. This is due to technical challenges in detecting circRNAs, as well as problems in genetically manipulating circRNAs without affecting linear RNA expression from their host genes. By numbers, so far, 10 circRNAs have been firmly associated with cardiovascular pathophysiology by experimental evidence in cell culture systems that serve to model aspects of cardiovascular disease. Of these, only 3 circRNAs have been studied *in vivo*, that is in animal CVD disease models or by decisive evidence from genome-wide association studies (GWAS) in human patient cohorts. Another 27 circRNAs

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have been implicated as biomarkers for CVD but have not been functionally investigated. Given that many, if not most, circRNAs have been suggested to affect the expression of linear mRNA from their host genes, many more circRNAs can be expected to be linked to CVD in the future. Therefore, we will review here all the CVD-linked circRNAs, but start out by highlighting milestones in the investigation of general circRNA biogenesis and molecular function. In these introductory passages (chapters 2–4) we distill general concepts with relevance for cardiovascular disease, as then discussed later in the review of studies on specific CVD-linked circRNAs (chapters 5.1–5.3). Throughout, the main focus is the review of studies profiling circRNA expression in cardiovascular tissues, of studies exploring cellular functions of circRNAs in cardiovascular physiology and disease, and of studies translating these data to human pathophysiology. In the review, one major question will be how to prioritize circRNAs during the investigation of cardiovascular disease pathways, in times when expression profiles of thousands of circRNAs are accumulating through RNA high-throughput sequencing efforts in tissues, cells or blood samples. A second important question will be how to interpret genetic polymorphisms associated with CVD risk from GWAS, for example when risk single nucleotide polymorphisms (SNPs) are found to locate in CVD risk genes. Third, we will discuss how the spliceosome mediates circRNA biogenesis, and how alterations in splicing patterns are linked to the onset of cardiovascular disease.

2. Classes of circular RNAs

Three major classes of circular RNAs can be distinguished based on the type of covalent linkage underlying circularization, based on the molecular machinery circularizing 5′ and 3′ ends and based on the typical subcellular localization of the circularized RNA products. The large majority of circular RNAs in eukaryotes are produced by the spliceosome. Thereby, the major U2-containing spliceosome is the major machinery that produces circular RNAs. 3 groups of circular RNAs are produced by the U2-containing spliceosome: a. 3′-5′-linked circRNAs that only contain exonic sequences, b. 3′-5′-linked EIciRNAs, which contain both exons and introns, and c. ciRNAs, which contain only intronic sequences and have been circularized by a 2′-5′ covalent phosphodiester bond [11–13] (Table 1, Fig. 1). For the biogenesis of all these three groups of circular RNAs, in a reaction termed “backsplicing”, the spliceosome uses existing canonical splice signals [3,13] for cutting out and circularizing internal parts of pre-mRNA sequences *in cis*. In the following, we describe the classes of circular RNAs in more detail. The accompanying Box 1 contains a short list of vocabulary that can be used as an entry point into the field of circRNA biology.

2.1. Spliceosomal, exon-containing (3′-5′)-linked circRNAs

The first and major class of circular RNAs is **3′-5′-linked circRNA** produced by backsplicing, a reaction whereby the spliceosome fuses a downstream splice donor to an upstream splice acceptor [6]. In the simplest case by this reaction a 5’→3′-linked circRNA is formed that consist of a single exon, where the end of the respective exon attacks its own start in the second transesterification reaction. circRNAs can, however also contain multiple exons, whereby intervening introns are spliced out in a subsequent step (Fig. 1). Exon-only circRNAs can exhibit a number of roles, ranging from regulating linear splicing, over binding proteins and modulating their activity, to binding and sequestering microRNAs, termed microRNA sponging. Since circRNAs are sequences of fused exons but do not display a 5′Cap and other upstream features necessary for ribosome entry to linear mRNAs, circRNAs are classically not translated. However, the small possibility exists that protein-coding potential is still exhibited, and this is a known function of a tiny subset of circRNAs. All these functions are described in detail in chapter 4.

2.2. Spliceosomal, exon- and intron-containing (3′-5′)-linked EIciRNAs

When one or several intervening intronic sequences are maintained during the biogenesis of a circRNA, these specialized forms are termed circular **exon-intron-containing circRNAs** (**EIciRNAs**) [14] (Fig. 1). In a typical cell, approximately hundred EIciRNAs exist. They are, thus, a minority compared to exon-only circRNAs. EIciRNAs are mostly nuclear and have been involved in stimulating RNAP

Table 1
Spliceosome-dependent circular RNAs. Key features of circular RNAs produced by the spliceosome in eukaryotic cells. n (estimated number of relevant circRNAs expressed from a typical mammalian genome; counted per host gene), n^{total} (total number of all predicted circRNA transcript isoforms) as deduced from mapping RNAseq reads during transcriptome profiling of different human organs/tissues/cell types.

Common features	• Formed by the canonical cellular spliceosome (U2-containing and operating on canonical splice sites) • Formed from both coding and non-coding genes • Single-stranded RNA • No free termini/no 5′ or 3′ ends (circular) • No 5′ Cap, no poly(A) tail • Stable because resistant to RNase R (3′-5′ exoribonuclease) • Mostly non-protein coding (irrespective of origin) • Functioning mostly in control of gene regulation
Exon-containing (3′-5′)-linked circRNAs	• Only-exon-containing, 3’→5′-linked, introns have been spliced out • Mostly cytoplasmic; few exceptional cases in the nucleus • n > 10000 (per host gene) • n^{total} = 140790 circRNA transcripts/isoforms in humans; listed in: http://www.circbase.org/cgi-bin/downloads.cgi
Exon- and intron-containing (3′-5′)-linked EIciRNAs	• Exon-and-intron-containing 3’→5′-linked circRNAs • Nuclear • n^{total} = 111 in human cells (HeLa); listed in Ref. [14]
Intron-only (2′-5′)-linked ciRNA	• Stabilized 2’→5′-linked excised introns, not containing exonic sequences • Nuclear • n > 100 • n^{total} = 103 in human cells (HeLa, hESC) [17] • As a subgroup of ciRNAs, sisRNAs are stable intronic sequences (can be linear or circular, nuclear and cytoplasmic, n > 9000)

Abbreviations: circbase (curated database for circular RNAs [35]), HeLa (human cervical cancer cell line), hESCs (cultured human embryonic stem cell line).