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circRNAs in Cancer

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Abstract

Exonic circular RNAs (circRNAs) are mostly generated from exons of protein-coding genes and, in many cases, are more abundant than the linear product from their hosting gene. Certain circRNAs are very abundant in the brain and in non-dividing cells; and many also show physiological- and tissue-specific expression. Moreover, recent work has demonstrated that some circRNAs are functional; and foremost, an important number of research articles have pointed a relation between cancer and certain circRNAs. In this review, we describe general advances in the field regarding circRNA biogenesis and functions in relationship with cancer. Also, we summarize some necessary precautions to work with circRNA that are particularly relevant to cancer-related studies.

MAIN TEXT

General features of circRNAs

Circular RNAs (circRNAs) are a highly abundant type of RNA which was recently rediscovered and displays widespread expression in the tree of life [1–5]. These recent findings have been possible mainly due to the development and widespread use of RNA sequencing technologies, in particular those that do not rely on poly(A) purification, as well as the development of specific algorithms for circRNA detection and quantification [6].

Most circRNAs are generated from complete exons of protein-coding genes. They are generated by a process named back-splicing in which a spliceosome utilizes 3' splice site that is upstream of the selected 5' splice site (see Figure 1a). These RNA species tend to include exons that are toward the 5' of the gene and that are flanked by longer introns [7–9]. The long introns that flank regions that will become circRNAs usually contain specific sequences that induce circRNA formation either by complementarity and/or by binding of circRNA-promoting factors (Figure 1b,c) [8–10].

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circRNAs have long half-lives due to the lack of free 3' or 5' ends, which makes them resistant to regular mechanisms of linear RNA decay. Neural tissues are particularly enriched in circRNA, due to both high production rates and accumulation of circRNA molecules [7,11]. Moreover, circRNAs are highly enriched in synapses and their levels are modulated by neuronal activity, suggesting specific roles in the brain [11,12]. Indeed, recent work from the Rajewsky lab demonstrated that one circRNA has a specific function in the mammalian brain [13].

At the molecular level circRNAs seems to work through several mechanisms. Some circRNAs regulate gene expression in *cis* by tuning mRNA production from the host gene [9]. Other circRNAs function in *trans*. For example CDR1 and *Sry* bind specific miRNAs and work as miRNA sponges and/or transporters [1,13,14], and circMbl appears to act as a decoy for the multifunctional protein MBL [9]. Still, other circRNAs might bind transcription factors [15], are involved in muscle development [16], or viral transcription [17,18] or have other functions. Importantly, recent reports demonstrated that a subset of circRNAs is translated in flies and mice [16,19]. However, these reports do not address the functionality of any of the circRNA-encoded proteins, so it is not yet clear what is the function of their translation.

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Despite their widespread expression in most tissues, circRNAs are expressed at low levels in immortalized cell culture lines [3,20,21]. This is likely due to low production, which is generally associated with cells and tissues with high duplication rates. Indeed, high division rates are inversely correlated with circRNA levels (see below and [21]). A few years ago, a comprehensive assessment of circRNA levels across several normal and cancerous tissues showed that circRNAs are generally less abundant in highly dividing cells, in particular in tumors [21]. This study demonstrated the existence of an inverse correlation between the relative abundance of circRNAs and cell proliferation, which was attributed to dilution of the circRNA population due to high duplication rates. In this study, the authors compared the circRNA levels to those of the mRNAs produced from the same gene, which ruled out simple transcriptional effects on the host gene [21]. Anti-correlations between circRNA steady-state levels and cell proliferation were demonstrated in samples from colon cancer and ovarian cancer patients and in patients with idiopathic pulmonary fibrosis, another disease related to differential cell proliferation [21]. An anti-correlation was also observed when tissues with different replication rates were compared. These results suggest the existence of a universal trend by which cells with high proliferative rates display low levels of circRNAs. This would insinuate *a priori* that circRNAs are unlikely to be involved in cancer; however, recent studies suggest that circRNA might have roles in different types of cancer [15,22,23]. Moreover, and independently of their putative role in cancer development and/or progression, circRNAs could potentially be powerful biomarkers for cancer given their long half-lives and resistance to common degradation pathways [24,25].

De novo generation of a cancer-promoting circRNA

The involvement of circRNAs in cancer could be explained by three different phenomena. The first one, explained in this section, is the generation of a toxic circRNA as a result of a

genomic translocation, as described by Guarnerio *et al.* [22] (see Figure 2a). In this study, the authors describe how a chromosomal translocation led to the generation of a novel circRNA that contributes to cellular transformation, promotes cell viability, and has tumor-promoting properties. They refer to this new type of circRNAs as fusion circRNAs or f-circRNAs. Guarnerio *et al.* describe a circRNA that is generated by the most recurrent translocation in promyelocytic leukemia, which involves the PML and RAR α genes. The authors show that this translocation induces the generation of a f-circRNA formed by the 5' head of PML exon 5 and the 3' tail of RAR α exon 6. They follow up this finding in different cancer cell-lines, and find different f-circRNAs and also different variants of them. For example, the MLL/AF9 aberrant translocation in the THP1 cell line gives rise to two distinct f-circRNA; and the EWSR1/FLI1 and EML4/ALK1 translocations give rise to other f-circRNAs in SK-NEP-1 sarcoma cells and in H3122 lung cancer cells, respectively. Given the low production rates of circRNAs and their very low levels in dividing cells [21], this example is likely to be an exception rather than a general rule. It will be interesting to evaluate the production rate of this particular circRNA in order to understand how this oncogenic circRNA is not diluted as the cancer progresses.

Downregulation (dilution) of an anti-proliferation circRNA

A second mechanism involves the downregulation of a specific circRNA that directly or indirectly exerts tumor suppression functions. This could happen at different stages of the disease. As circRNAs are long lived and their concentrations are very sensitive to division rates, this mechanism could be downstream of initial events that increase proliferation (see Figure 2b, left panel). Alternatively, a more specific regulation (i.e., cleavage induced by a small RNA) could diminish circRNA levels by degrading the molecule (see Figure 2b, right panel). The latter might not be as common, as until now there is only one example of a miRNA that has complete complementarity and can bind (and degrade) a specific circRNA [1,13,14].

Several examples in which downregulation of a specific circRNA is linked to an increase in cell proliferation have been described in the literature. For example, recent work suggested a role for circITCH in different cancer types. Li *et al.* found that this circRNA is significantly downregulated in esophageal squamous cell carcinoma samples [23]. Interestingly, circITCH possesses several miRNA-binding sites, suggesting that it might work as a sponge, transporter, and/or regulator of those miRNAs. Moreover, overexpression of circITCH modulates cell growth and suppresses tumor growth [23]. These effects might be due to misregulation of the Wnt/ β -catenin pathway. Another study suggested a role of the same circRNA in lung cancer [26]. In this study circITCH was found to be present at lower levels in lung cancer tissues than in normal tissues. The lower expression might be due to lower transcription of the locus, as the *ITCH* mRNA is also significantly downregulated in lung cancer patients. Interestingly, the authors found that overexpression of circITCH had specific effects in a model of lung cancer mediated by the Wnt/ β -catenin pathway, and circITCH might regulate the activity of oncogenic miR-7 and miR-214 to inhibit cancer progression. In addition, Huang *et al.* [27] found decreased levels of circITCH in colon and rectal cancer tissues and a recent report linked circITCH downregulation to hepatocellular carcinoma [28]. In the latter study, the authors also found two SNPs in the circRNA sequence that

correlate with this disease. Although these reports are interesting, they are mainly based on correlation and overexpression studies; thereby the function and mechanism of this circRNA must be characterized in more detail. Nevertheless, these studies do clearly illustrate the potential of circITCH as a biomarker for cancer.

Another circRNA, circFOXO3, has also been linked to cell proliferation [29]. Interestingly, similar to findings regarding circITCH, low levels of circFOXO3 seem to be associated with an increase in cell proliferation [29]. By overexpressing circFOXO3 and performing silencing and purification experiments, the authors propose a model in which circFOXO3 forms a ternary complex with p21 and CDK2. In this model, circFOXO3 delays the transition along the cell cycle. Thus, the absence of the circRNA increases cell cycle entry. Interestingly, circFOXO3 also seems to regulate cell growth by an independent mechanism that involves sponging of specific miRNAs that regulate the production of *Foxo3* mRNA [30].

Two studies suggest roles for circHIPK3 in cell proliferation and cancer [31]. Zheng *et al.* found that this circRNA is involved in cell growth [31]. Briefly, the authors identified potential cancer relevant circRNAs by sequencing and characterizing a panel of normal and cancerous tissues. Among the circRNAs detected was circHIPK3, which is significantly upregulated in liver cancer. Interestingly downregulation of this circRNA inhibits growth of Huh7, HCT-16 and HeLa cells in culture. The authors propose that this effect is due to the sponging activity of several tumor suppressive miRNAs including miR-124. More recently, Li *et al.* implicated circHIPK3 in bladder cancer progression [32]. Contrary to the reported changes in liver cancer, the candidate circRNA is downregulated in the bladder cancer lines, suggesting that dilution of the circRNA might be related to the development of the disease. Supporting this hypothesis, overexpression of the circRNA in bladder cancer cell lines reverts some invasive and proliferating properties of these cancerous cells, whereas downregulation of circHIPK3 increases their migration and invasive capabilities. Mechanistic studies suggest that this circRNA acts by regulating the levels of miR-558, which is highly expressed in human bladder cancer and is known to regulate the expression of heparanase [32]. While reports on circHIPK3 might seem contradictory, their differences could be due to different factors. First, the two studies analyze two completely different data sets and focus on different types of cancer cells. Second, even when they claim that the observed association between circHIPK3 levels and cancer features is due to sponging activity of this circRNA, they identify different candidate miRNAs and putative targets. This is due to the use of different cell systems as well as different computational approaches. Finally, they perform opposite functional assays; while Zheng *et al.* performs knock-down experiments, Li *et al.* performs overexpression studies.

The cases described above suggest that an increase in proliferation of cancer cells indeed dilutes key circRNAs and results in advancement of the disease. In this context, it might be important to determine how circRNAs are distributed among daughter cells following cell division and whether possible asymmetric distribution of the circRNA in daughter cells may result in different proliferation rates (see Figure 2b).

Upregulation of the levels of a pro-proliferation circRNA

A third potential way in which circRNA misregulation could contribute to cancer involves upregulation of a pro-tumorigenic circRNA (Figure 2c). Although less intuitive, this type of mechanism has been proposed for a handful of circRNAs and could be analogous to that by which oncogenic proteins with very low abundance and/or short half-lives contribute to cancer. Yang *et al.* showed that circAmotl1 is highly expressed in cancerous samples and cell lines, and can promote cell proliferation [15]. Despite the evidence, it is difficult to rationalize how high circRNA levels could be maintained over time in cells that divide so frequently unless cancer cells express a disproportionate amount of this circRNA. In this context, it is surprising that the phenotypes are observed upon overexpression of the circRNA, which should increase during proliferation. The authors propose that the mechanism involves the translocation of c-myc to the nucleus [15].

In addition, two different studies suggest roles of circMYLK and circTCF25 in bladder cancer [33,34]. In both cases the authors postulate sponging activity of miRNAs by the upregulated circRNA [33,34]. Similarly, circABCB10 [35] and circBANP [36], which are upregulated in breast and colon cancer samples, respectively, might promote cell proliferation in those cancers. Other reports postulate roles for several circRNAs in cancer initiation and/or progression including circrna_001569 (circABCC1) [37], has_circ_0067934 (circPRKCI) [38], circZNF292 [39], hsa_circ_0013958 (circACP6) [40], circRNA_100290 (circSLC30A7) [41], and circSMARCA5 [42]. Although the roles of these circRNAs in proliferating cells have not been tested independently, reported experiments suggest that this type of circRNA might be extremely useful as biomarker of the disease (see below).

circRNAs as cancer biomarkers

circRNAs are highly stable molecules, hence it is not surprising that circRNAs are highly enriched in comparison to linear mRNAs, miRNAs, and other RNA types in whole blood, plasma, and platelets [43]. In particular, the high amounts of circRNAs in blood derive from their stability in platelets and other fractions that contain intact circRNAs but only fragmented mRNA and rRNAs [43]. Because of this, it was early speculated that circRNAs could be excellent biomarkers for disease. Given their stability and cell specificity, even small amounts of circRNAs that originate from cells released into the bloodstream from a localized tumor would be detectable over time. This promising role of circRNAs as cancer biomarkers requires, however, that specific circRNAs are differentially expressed upon transformation or in early stages of specific cancers. This could occur, for example, if the circRNA is produced from a genomic region that was amplified in that specific context. Alternatively, the disappearance of a very abundant circRNA could also be utilized to biomark disease. Over the last few years several studies have tested the relevance of circRNAs as biomarkers of different types of cancer (see references [24,25,44–46] for more details). These results still need to be corroborated in larger numbers of samples and patients, but they demonstrate the promise of circRNAs as biomarkers for cancer.

Technical problems in circRNA research

Working with circRNAs might seem analogous to studying any other type of coding or non-coding RNA; however, there are several obstacles for circRNA research that are usually overlooked and that are particularly relevant to cancer-related studies. Those obstacles mainly derive from the fact that the majority of the circRNA sequence is shared with the mRNA generated from the host gene. Hence, circRNA identification, quantification, and validation, as well as overexpression and silencing strategies, all rely on the specific back-splicing junction and are particularly sensitive to biological and experimental artifacts [47]. When performing circRNA research care must be taken to ensure (1) thorough validation, (2) accurate quantification and (3) careful confirmation of any experimental manipulation of the levels of the specific circRNA.

circRNA validation—The presence of a putative circRNA junction detected by qPCR does not guarantee the existence of a circular RNA. This is because these atypical splicing junctions can originate from linear RNAs if, for example, two consecutive exons have very similar sequences or if a given RNA is the product of trans-splicing that leads to exon duplication or reshuffling [6]. Moreover, template-switching activity from the reverse transcriptase can also result in cDNA with duplicated sequences. Therefore, circRNA validation is a crucial step in any circRNA study. Stringent circRNA validation generally requires the use of the 3' exonuclease RNase R and Northern blots, which are a more accurate way to fully characterize RNA species containing a given exon or exons than RT-qPCR [7]. Although RT-qPCR is a better quantitative method, it does not allow unequivocal identification of *bona fide* circular RNAs. Importantly, and as the RNase R treatment introduces a large change in the concentration and composition of the RNA mixture to be subjected to RT-qPCR, adequate internal controls (i.e., spike-in of known amounts of foreign RNA) are necessary to evaluate the RNase R sensitivity of a specific circRNA candidate [7]. Another essential step for circRNA validation is the corroboration of the existence of the backsplicing junction by Sanger sequencing.

circRNA quantification—Quantification of circRNAs is not easy, and quantification of a specific junction must be performed by RT-qPCR. Therefore, primer pairs must be tested thoroughly for linearity. Also, another important issue is the RNA-seq protocol and pipelines to be used [48]. Because circRNA identification and quantification relies on a unique junction, the counts of circRNA are low even for very abundant circRNAs. As longer RNA-seq reads increase the detection power of circRNA identifying algorithms, long reads and deep RNA-sequencing runs increase the power to detect circRNAs. Moreover, different computational pipelines vary in their sensitivity and accuracy for quantifying circRNA, adding an additional layer of complexity to the problem of circRNA quantification [48]. Given the particular characteristics of the circRNA junction, circRNA identification and quantification requires specifically designed bioinformatics pipelines. Corroborating differentially expressed circRNAs can be truly problematic and many times should include the results of different pipelines. Furthermore, statistical analysis of this large amount of data needs to be carefully performed to assess correctly the significance of the tests used. Consequently, differences in levels of circRNA expression should be corroborated also by

qPCR analysis. This is central for the cancer research field when comparing the levels of circRNAs in cancer vs non-cancer samples.

Manipulating circRNA levels—Last but not least, manipulating circRNA levels is also problematic. To deplete cells of circRNAs, it is common to use short-hairpin (sh) RNAs that mediate degradation through the RNA interference pathway. However, as the shRNA by definition has to be in large part complementary to the linear mRNAs produced from the genes generating circRNAs there is a clear risk that a shRNA targeting a circRNA will also modulate the levels of the linear mRNA encoded by the same gene. This makes it difficult (if not impossible) to dissociate effects of linear and circRNAs produced from the same locus. In addition, shRNAs can induce off-target effects. Hence, it is highly recommended to employ several shRNAs by shifting the target sequences a few bases. This is *per se* a difficult issue, as the backsplicing junction is the only sequence which is specific for the circRNA. It is essential, however, to utilize different shRNAs, and to rule out experimentally and computationally putative off targets. CRISPR-based approaches to inhibit circRNA production might be cleaner than those involving shRNA. However, CRISPR approaches cannot be used to distinguish *cis* and *trans* effects of the candidate circRNA, as deletion of flanking intronic sequence might likely impact mRNA production as well.

In addition, overexpression of circRNAs is also challenging. This is because transfected plasmids tend to form concatemers that resemble circRNAs but are linear molecules [49,50]. In some cases, this problem can be overcome by choosing intronic sequences that minimize concatemer-formation. Also, strategies that involve the transfection or integration of few or single plasmid molecules are preferable. Hence, in all cases overexpression experiments must be validated by Northern blot. In sum, circRNA work requires very specific considerations, given the unique nature of these molecules.

Future Prospects

CircRNAs have emerged as a new type of molecule with intriguing molecular functions and potential importance as biomarkers. The number of articles showing at least some relation between specific circRNA and cancer features has increased during the past two years (see Table 1). Nevertheless, it is not clear yet what is the exact contribution of circRNAs to cancer generation and progression. In this context, the current challenge for the field is to uncover the mechanisms by which specific circRNAs can work as cancer promoting or suppressing agents. This is not easy, as circRNAs do not seem to act through a common mechanism, but to have different molecular modes of action. Therefore, advances in the general field of circRNA research will be key to unravel their potential importance in cancer. Furthermore, and in order to test specific hypothesis, the field will require the development of new tools to be able to manipulate circRNAs dynamically *in vivo*. These could involve CRISPR variants targeting RNA molecules or the identification of small molecules that could modulate the formation, stability or function of specific circRNAs. Hence, studying roles of circRNAs in cancer is an exciting and likely fruitful enterprise. In addition, the use of circRNAs as cancer biomarkers can soon be exploited for early screening of certain types of cancer, which could dramatically increase the chances of survival.

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Figure 1. General mechanism of circRNA biogenesis

A. Regular splicing (red arrows) and back splicing (blue arrows) give origin to mRNA and circRNA, respectively. Both processes are co-transcriptional and can compete between each other. circRNAs have a specific junction, unique for this molecule (blue dashes). CircRNAs can also contain exons which are joined by regular splicing and have a junction shared with the linear form (red dashes). **B.** Back splicing can be induced by proteins that bind to specific regions in the flanking introns and help to get the circularizing exons together. **C.** Exon circularization can also be induced by inverted complementary sequences (yellow arrows) in the flanking introns that stabilize and bring together the exons involved in back splicing.

Figure 2. Cancer and circRNAs

A. *De novo* generation of a cancer-promoting circRNA by chromosome translocation: Two complementary inverted sequences (yellow arrow) gets together after chromosome translocation and promote the generation of a new oncogenic circRNA. **B.** Negative correlation between circRNA and cancer features: If a circRNA that has some cancer-suppressor activity gets diluted due to cell division, cells with critically low concentration of this circRNA might have an increased proliferation rate (left panel). Alternatively, miRNAs that are deregulated during cancer might target this particularly circRNA and promote carcinogenesis as a result (right panel). **C.** Positive correlation between circRNA and cancer features: Some circRNAs have been pointed to be increased in cancer; therefore, they have to avoid dilution due to high cell proliferation ratio. This could be analogous to the way in which oncogenic proteins with very short half-lives contribute to cancer.

Table 1

Name and genome location of circRNAs in this review.

CircRNA name given in paper	Alias in circBase	Gene symbol	Position	Reference
has_circ_0067934	has_circ_0067934	PRKCI	chr3:170013698-170015181	38
hsa_circ_0013958	hsa_circ_0013958	ACP6	chr1:147131074-147131890	40
circRNA_100290	hsa_circ_0013339	SLC30A7	chr1: 101372407–101379362	41
circSMARCA5	hsa_circ_0001445	SMARCA	chr4:144464662-144465125	42
circFOXO3	Inferred hsa_circ_0006404	FOXO3	Not provided, Inferred: chr6:108984657-108986092	29, 30
circHIPK3	hsa_circ_0000284	HIPK3	chr11:33307958-33309057	31, 32
circTcf25	hsa_circ_0041103	TCF25	chr16:89962397-89967202	34
circMylk	hsa_circ_0002768	MYLK	chr3:123471177-123512691	33
circABCB10	hsa_circ_0008717	ABCB	chr1:229665945-229678118	35
circBANP	Not provided/not traceable	BANP	Exon 5–11 of BANP gene. Inferred: chr16:87987005-88017865	36
circRNA_001569	hsa_circ_001569	ABCC1	chr16:16101672-16162159	37
circITCH	Not provided/not traceable	ITCH	Exons 6–13 of ITCH gene. Inferred: chr20:32996456-33033266	23, 26, 27, 28
Cdr1AS	hsa_circ_0001946	CDR1	chrX:139865339-139866824	1,13,14