Circular RNA maps paving the road to biomarker development?

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In recent years, the RNA world has gained increasing attention due to the discovery that in addition to the classical mRNAs, tRNAs, and rRNAs, various other functional mRNA forms exist including miRNAs, lncRNAs, piRNAs, siRNAs, tmRNAs, sRNAs, tiRNAs, eRNAs, snoRNAs, snRNAs, and other non-coding RNAs, which seem to play various roles under physiological and pathophysiological conditions. In particular, miRNAs have now been acknowledged to be of importance in many disease pathologies and are now being considered as diagnostic and even therapeutic tools.

A recent addition to this family of diverse RNA molecules is the increasing number of various forms of circular RNAs (circRNAs). Although the presence of circular RNAs has already been noticed several decades ago in eukaryotes [1], they have been considered for a long time as transcriptional noise [2]. The extensive use of next-generation sequencing technologies together with advanced bioinformatic approaches has shown the presence of an abundant number of this diverse class of RNA molecules in various cell lines and tissues and across different species [3] (Fig. 1).

Most circRNAs are produced during the backsplicing of exons, introns, or both, a process which is generally catalyzed

by either the spliceosomal machinery or by group I and II ribozymes or by exon skipping [4]. Unlike linear RNA, the 3' and 5' ends in circRNA normally present in an RNA molecule have been joined together, forming a covalently closed continuous loop with a backsplicing junction, which makes them distinguishable from their linear counterparts. The circular loop structure also prevents degradation by RNA exonucleases, thus rendering these molecules highly stable [5]. circRNAs can contain one to many exons, and/or introns, and multiple circRNAs can be produced from a single gene. Most circRNAs derived from exons tend to be localized in the cytoplasm although the mechanisms of nuclear export are not clarified [5, 6]. circRNAs derived from introns, or exons and introns can be found in the nucleus [7].

The expression of circRNAs is not tightly bound to the expression levels of the linear transcript from which the circRNA is derived, indicating that expression of circRNA is regulated and that the spliceosome must be able to discriminate between forward splicing, i.e., canonical linear splicing and backsplicing [8]. While the levels of many circRNAs are much lower than their linear transcripts, in other cases, circRNA levels surveil over linear transcript levels or are even the only RNA species detectable. In human fibroblasts, it was estimated that circRNAs were derived from approximately 14% of actively transcribed genes, and that for some genes, circRNAs were more abundant than the corresponding linear mRNAs [3]. Analyses of RNA-seq data from the ENCODE project indicated that approximately 7000 human circRNAs showed an abundance of at least 10% of the transcripts from the corresponding genomic loci [9].

Key factors which seem to count for circRNA levels in cells and tissues are circRNA stability and the proliferative status of the cells in which they are expressed. Under the assumption of a steady transcription rate for a gene, linear mRNA products are expected to be continually degraded over time while circRNAs

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Fig. 1 Different forms of circRNAs are generated in the nucleus containing exons, exons and introns, or only introns. CircRNAs containing introns are mainly localized in the nucleus where they contribute to posttranscriptional regulation by binding to elongating RNA Pol II thus promoting transcription. CircRNA formation can compete with pre-mRNA splicing, which can alter the composition of processed mRNA due to the lack of specific exons from the pre-

mRNA resulting in lower levels of linear mRNAs. Exonic circRNAs can translocate to the cytoplasm by so far unknown mechanisms. CircRNAs can serve as a hub to bind miRNAs and affect their biological functions. CircRNAs can interact with proteins, trapping them in the cytosol or preventing their translocation into other organelles. Some circRNAs might translate into proteins

persist, thus allowing accumulation of circRNAs leading to an increased ratio of circRNA to mRNA. Cell division in proliferating cells or cell death would then diminish the circRNA/mRNA ratio, an observation which has been made in particular in tumor cells [10]. On the other hand, circRNAs would build up in terminally differentiated cells that rarely turn over as has been found for example in the brain [11].

Apart from this passive way of regulating circRNA abundance, circRNA levels can also be actively regulated involving RNA-binding proteins (RBPs) such as quaking. This RBP dynamically regulates the formation of circRNAs during human epithelial-mesenchymal transition [12].

On the other hand, targeted cellular export via exosomes or microvesicles has been described as a way to specifically decrease the cellular levels of these stable RNA molecules [13]. circRNAs, but not their linear RNA counterparts, have been found enriched in exosomes for example derived from tumor cells or tissues [14, 15].

The increasing attraction of circRNAs derived not only from their abundance in the eukaryotic transcriptome but also

from recent findings that these circles might exert various important biological functions related to the control of mRNA and protein levels at several instances.

Great interest was raised by the findings that some circRNAs could function as miRNA super sponges which are able to trap a large amount of specific miRNAs thus affecting gene expression levels and cellular functions [16]. To date, however, only two of these super sponges have been identified, including ciRS-7/CDR1as which harbors > 70 conserved binding sites for miR-7 and SRY, a testis-specific circRNA, which contains 16 binding sites for miR-138 [16]. Other circRNAs like circHIP3K were shown to contain binding sites for multiple miRNAs [17]. However, the majority of circRNAs identified to date seems to contain much lower numbers of miRNA binding sites which might not be sufficient to effectively trap miRNAs.

In addition to miRNA, circRNAs have also been suggested to act as protein sponges. circFOXO3a can interact with stress-related transcription factors such as HIF1 α , ID1, or E2F1 in the cytoplasm thus preventing them from nuclear translocation [18].

The muscleblind (MBL/MBLN1) protein in flies and humans can control its own protein level since it promotes the production of circMBL which has multiple binding sites for MBL, thus trapping this protein when in excess [19]. Moreover, this example shows that exonic circRNAs can act as traps for their parental mRNA due to a competition between circularization and splicing [19]. Furthermore, when backsplicing occurs, the internal exons are removed, causing alternative splicing and a shorter transcript. In addition, circRNAs can contain the translation start site as has been shown for the mouse formin gene, thus leading to an mRNA trap which leaves a noncoding linear transcript and thereby reduces the protein level encoded by the parental gene [20].

Moreover, intronic or mixed circRNAs which are found in the nucleus can promote Pol II transcription by interacting with the Pol II machinery [7]. The increasing evidence that circRNAs interact with the transcriptional machinery provides not only new insights into the mechanisms of gene expression regulation in cells, but also indicates that circRNAs might be involved in physiological processes as they are induced during human development [21].

Interestingly, there is also some recent evidence that certain circRNAs can be translated, thus challenging the view that circRNAs belong to the family of noncoding RNAs [22–24].

However, the importance of these findings is still to be elucidated and might not be a general phenomenon.

Subsequently, there is an increasing number of studies suggesting the involvement of circRNAs in the pathogenesis of different diseases such as cardiovascular diseases [25], neurodegenerative diseases [26], and cancer [27]. These latter findings raised the idea that circRNAs potentially contribute to pathological states and/or might be useful as biomarkers of disease.

Because circRNAs exhibit tissue- and developmental-specific expression [3, 5, 28, 29], comprehensive detection and quantification of circRNAs are essential not only for exploring their biological functions, but also to set center stage for the development of future biomarkers.

In the current study, Maass et al. [30] characterized the circRNA profiles from different human cell types and organs including vascular and blood cells. In addition to the annotation of more than 1800 new circRNAs, the authors further provide large support for the notion that circRNAs are highly tissue-specific. Interestingly, when mesenchymal stem cells from fat tissue were differentiated to different cell types; each cell type showed a vastly unique pattern of circRNAs although all tissues were derived from the same donor. In many cases, tissue-specific isoforms of circRNAs derived from the same parent gene were observed. Moreover, in various circRNAs, in particular, from tissues with high circRNA abundance such as platelets, a high circRNA to linear transcript ratio was observed. While the exact importance of this finding is not yet clarified, other data in this study demonstrated enrichment of circRNAs derived from pathophysiological

relevant genes involved in adenosine deaminase-deficient, severe combined immunodeficiency (ADA-SCID) and in Wiskott-Aldrich syndrome (WAS) compared to healthy controls. These findings suggest that circRNAs could act as biomarkers for those, but also for other diseases. In fact, several studies attempt to provide evidence that circRNAs could act as biomarkers for example in cancer (for reviews see [31–33]).

However, in order to be able to suit as new biomarker, several points need to be addressed in more detail that are relevant for a sensitive, specific, robust, and reproducible assessment of tissue or disease-specific circRNAs in diverse laboratory settings. Only then, they can be used for validation in clinical trials and application in patient diagnostics.

One major aspect is the analytical soundness of the methods that includes high analytical specificity and sensitivity and high accuracy and reproducibility of test results within and between labs [34, 35].

This applies for single circRNA as well as for circRNA patterns that are used for tissue or disease differentiation. If circRNAs are to be quantitatively assessed in bodily fluids, high recovery and dilution linearity in different matrices as well as robustness against potentially disturbing factors are required. All these items seem to be self-understanding but require highly standardized processes and effective quality controls at many levels.

Thus, appropriate controls have to be established to estimate the comparability and efficiency of all analytical processes such as circRNA isolation, reverse transcription, and quantification as well as for normalization and interpretation. Beyond internal quality controls, external ring trials will support laboratories to guarantee and document the required quality levels of the diagnostics as currently done for several other molecular markers [36, 37].

Although high stability of circRNA is assumed, the potential influencing factors of tissue and blood collection and handling have to be investigated in detail to define standardized operating procedures for robust and reproducible analytics; the conditions of sample collection, devices, and procedures applied and the comparison of sample transport, handling, processing, and storage may contain major issues that influence test results considerably. Are specific collection tubes with RNA-stabilizing reagents necessary? Are there specific requirements for tissue or cell processing? Is serum and plasma equivalent? Which differences in transport time and temperatures are acceptable? Experiences with miRNA have shown that even if they are more stable than mRNA, many questions regarding analytical differences and pre-analytical influencing factors are still to be clarified to enable their robust and reproducible quantification and an acceptable lab-to-lab comparability [38].

Regarding the donors of the materials, the intra- and interindividual variability within the groups of healthy donors and patients with different types of diseases, the

biological variations over time, the dependency on age and gender, the influence of medication, activity, life style etc. have to be addressed before the introduction of new biomarkers in the diagnostic process [34, 39]. All these issues are far less fancy than the discovery of new patterns of biomarkers in a few well-defined samples. However, their thoroughly and detailed examination is crucial to create a reliable basis for the subsequent clinical validation.

In patient care, biomarkers may be used for different clinical questions: (i) the (early) diagnosis or exclusion of a disease, (ii) the characterization of the severity of a disease, (iii) the estimation of the prognosis, (iv) the stratification of a patient for a specific therapy, (v) monitoring the effectiveness of a therapy, (vi) monitoring the disease state after primary therapy, and (vii) the early detection of recurrent or progressive disease.

For all these different questions, biomarkers have to be tested and validated in appropriate groups of patients in sufficient numbers and with appropriate controls. Requirements are already high for one-time investigations to evaluate single or patterns of biomarkers for the application in disease screening, diagnosis, or estimating prognosis. For monitoring purposes, the definition of disease and therapy conditions, the determination of time points for sample collection and biomarker measurement, the assessment of outcome and comparison with established diagnostic procedures as well as the performance of prospective trials and the development of meaningful algorithms are quite challenging endeavors [34, 39]. Nevertheless, these studies are necessary to create the evidence-based knowledge to improve diagnosis and patient care with such promising new diagnostic tools as circRNAs.

Finally, there are plenty of additional approaches like genetic and epigenetic characteristics on circulating DNA—which is referred to as liquid biopsy in cancer diagnostics [40]—all sorts of different RNA subtypes that are present in various cellular and extracellular blood compartments, peptides, proteins, lipids, glycation, and metabolite patterns that are recommended for diverse clinical questions. In the end, the quality of the method itself, the feasibility of the application as an easy-to-use, robust, reproducible, cost-efficient, and highly scalable tool, and the clinical relevance with respect to higher benefit for the patients and improvement of health care economics will decide on the implementation of new biomarkers in the future. The present work of Maass et al. highlights the promise of circRNA as tissue and disease specific markers. Further studies will show whether these markers will make their way into clinical diagnostics.

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