GENETICS

Are circRNAs involved in cancer pathogenesis?

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In a paper published recently in *Cell*, Guarnerio *et al.* suggest that circular RNAs derived from cancer-associated chromosomal translocations have an oncogenic role; however, the experimental approach that the authors used was inadequate to generate sufficient evidence to prove this role, calling their study into question.

Refers to Guarnerio, J. *et al.* Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. *Cell* **165**, 289–302 (2016)

Circular RNAs (circRNAs) can be generated from precursor mRNAs (pre-mRNAs) that are transcribed by RNA polymerase II as a result of noncanonical splicing, whereby the 3′ and 5′ ends of all or part of the linear mRNA molecule are covalently joined to form a closed continuous loop (FIG. 1). The existence of circRNAs was discovered over 20 years ago, and, in general, these nucleic acids have been considered to be byproducts of imperfect splicing[1–3]. Excellent reviews on circRNA biogenesis and the regulation of this process have been published in the past 2 years[4,5]. Of note, studies have revealed that several types of circRNAs can be produced from a single gene[4,5]. In addition, thousands of circRNAs have been shown to accumulate in the brain, and several hundred are upregulated during epithelial-to-mesenchymal transition (EMT) in human cells[4,5]. No correlation has been found between the levels of mRNAs and their relative circRNAs[6]. Interestingly, however, the splicing factor muscleblind (homologous to muscleblind-like protein 1 in humans) is capable of binding to and increasing circRNA production from its own pre-mRNA[7], suggesting that circRNA biosynthesis can be a mechanism to regulate translation via competition with canonical splicing of co-transcribed pre-mRNAs. In a similar manner, the product of the Quaking (*QKI*) gene, another regulator of pre-mRNA splicing and a known tumour suppressor, is upregulated during EMT; when expressed at high levels in mesenchymal cells, this protein seems to enhance the formation of circRNA by juxtaposing circularized exons[8].

Researchers have postulated that circRNAs are produced from >10% of expressed genes[4,5]. Despite arising mostly from protein-coding genes, evidence that circRNAs are translated is scarce and, therefore, these oligonucleotides are classified as noncoding RNAs. Furthermore, most circRNAs are expressed at very low levels compared with the expression of protein-coding mRNAs, suggesting that they lack functional activity. Nonetheless, certain circRNAs clearly have important functions, for example, in the brain and testis[9]. In particular, ciRS-7 is a well-characterized circRNA that is generated from an antisense transcript of *CDR1* (encoding cerebellar degeneration-related autoantigen 1) and is highly expressed in both the mouse and human brain[9]. This circRNA contains at least 63 binding sites for the microRNA miR-7, but is resistant to miRNA-mediated degradation; therefore, ciRS-7 functions as a 'sponge' for miR-7, and has been shown to decrease miR-7 activity, thereby increasing the expression levels of miR-7 targets[9]. Nothing is known, however, regarding the possible roles of circRNAs in cancer and other diseases.

In a recent publication in *Cell*[10], the authors proposed an oncogenic role of circRNAs derived from fusion genes (f-circRNA) that result from cancer-associated chromosomal translocations. The generation of f-circRNA from genes involved in cancer-associated translocations is not surprising, considering

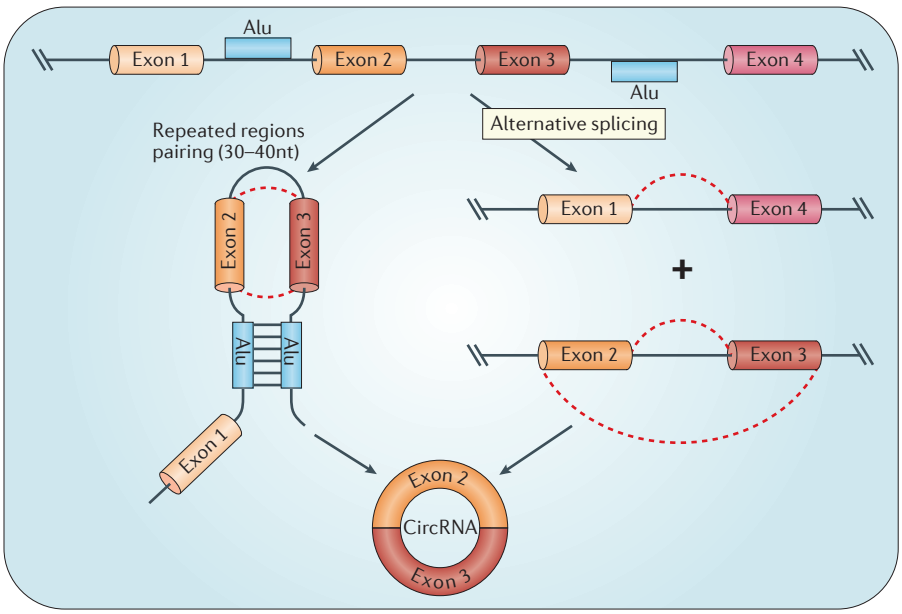

Figure 1 | **Biogenesis of circRNAs.** The presence of repeated sequences (such as Alu elements) is a catalyst in the formation of circular RNAs (circRNA; left). Use of an incorrect splice acceptor site, or the removal of several consecutive exons in an alternatively spliced transcript can also lead to the creation of circRNAs (right). nt, nucleotides.

that circRNAs seem to be produced from more than 10% of all expressed genes[4,5]. Rather, the key question is whether such circRNAs contribute to cancer development. The researchers state that gene-fusion-derived f-circRNAs "contribute to cellular transformation, promote cell viability and resistance upon therapy, and have tumour-promoting properties in *in vivo* models" (REF. 10). This proposition and supporting data, however, warrant critical analysis.

Firstly, although the results of the experiments performed do indeed suggest that some f-circRNAs are expressed in leukaemia cells harbouring different chromosomal translocations, the quantitative amounts and significance of f-circRNAs expression in relation to the expression of the fusion-gene products are not presented[10], thus precluding assessment of the relevance, if any, of the findings.

Next, the authors attempted to prove that f-circRNAs contribute to tumorigenesis by carrying out transfection experiments in cultured mouse embryonic fibroblasts (MEFs) that do not express the parent fusion proteins[10]. Such models were used extensively three to four decades ago to demonstrate oncogenic potential, for example, of the activated *RAS* gene and a few other oncogenes. The leukaemogenic role of the fusion genes investigated in the study[10] has never been shown in this MEF transformation assay, raising important questions concerning the rationale of the transfection experiments described in this report. To my knowledge, no one has ever demonstrated the leukaemogenic potential of oncogenes derived from gene fusions using the MEF transformation assay, and a review of the literature from the past 40 years does not reveal one example. Nevertheless, the authors claim that transfection of MEFs with f-circM9, a f-circRNA derived from the *MLL–AF9* fusion that results from the t(9;11) chromosomal translocation, increases foci formation in the assay, despite the fact that the *MLL–AF9* fusion is found almost exclusively in acute myeloid leukaemia cells and is not known to transform MEFs. Notably, the authors did not use the *MLL–AF9* fusion oncogene as a control — perhaps because they knew it would not result in transformation (despite the fact that it should produce f-circM9). Why then should a f-circRNA produced from the *MLL–AF9* fusion transform MEFs, when the fusion gene itself is unlikely to be transforming in these cells? Indeed, the capacity of f-circM9 to induce transformation of MEFs is surprising. What is the proof that transformation is caused by f-circM9 and not by something else, for example, by *RAS* mutation? On the basis of the data presented[10], the number of colonies formed by cells transfected with a mutated form of f-circM9 does not seem to be much lower than that produced by MEFs transfected with wild-type f-circM9; moreover, the assay is quite prone to generating artefacts.

In subsequent experiments involving haematopoietic-stem-cell (HSC) transformation and transplantation into mice, none of the mice developed leukaemia in the 3 months following transplantation of cells transfected with f-circM9. Thus, the authors concluded that the presence of f-circM9 alone is probably not sufficient to trigger tumorigenesis. The researchers then attempted to show that the identified circRNAs do something, and decided to transfect the mouse HSCs that had previously been transfected with *MLL–AF9* with expression vectors encoding either f-circM9, or mutated f-circM9 (as a control). As shown in Figure 3D/E of their paper[10], HSCs expressing f-circM9 together with the MLL–AF9 fusion protein displayed an increased ability to proliferate and form colonies in serial plating assays, compared with the parental HSCs expressing only the MLL–AF9 fusion protein, or cells expressing MLL–AF9 and mutated f-circM9. Here, the *Cell* paper gets very confusing! The authors tell us that the *MLL–AF9* fusion generates circRNA (f-circM9) intrinsically, but nevertheless co-transfect cells with *MLL–AF9* (which causes leukaemia) and f-circM9 (which does not cause leukaemia), and observe more leukaemogenesis in *in vivo* transplantation experiments, as demonstrated by spleen enlargement and higher proportions of leukaemic cells in the bone marrow and spleen compared with mice transplanted with cells co-transfected with *MLL–AF9* and mutated f-circM9, but only when transplanted at a "limiting rate"(REF. 10). They also found that the circRNA was protective against chemotherapy[10].

As the *MLL–AF9* fusion generates f-circM9 anyway, do these results mean that the more f-circM9 is expressed, the greater the leukaemogenic potential? Where is the proof? How much f-circM9 is derived from *MLL–AF9* fusion, and how much is the result of the f-circM9 transfection? Indeed, the authors failed to quantitate how much f-circRNA was generated in the HSCs they transfected with only the MLL–AF9 expression vector, or how much of f-circRNA expression was derived from the f-circM9 vector in the co-transfected cells. Thus, conclusively demonstrating that f-circRNA derived from *MLL–AF9* contributes in some way to leukemogenesis is quite difficult because f-circM9 expression alone does not cause leukaemia, as mentioned by the authors, whereas expression of MLL–AF9 does, and because the *MLL–AF9* fusion gene itself, as stated by the investigators, can also be expressed as a f-circRNA. This study is extremely confusing and unclear, and the data are not convincing because they are difficult to deconvolute and could simply be artefactual.

In my opinion, the paper does not convincingly show that circRNAs contribute to tumorigenicity; in fact, it shows just the opposite. The construction of transgenic mice expressing different levels of the *MLL–AF9* f-circRNAs could conclusively address the question of whether circRNAs of the *MLL–AF9* fusion gene are leukaemogenic, but this experiment was not performed.

What is surprising is that this paper was published in *Cell*, which is widely considered to be one of the top journals in the fields of molecular, cell, and cancer biology. In this regard, editors and referees have an important role in ensuring the scientific data published in the primary literature is sound, and that the findings reported are substantiated by appropriately controlled experiments and robust data — that is, they must resist the 'splash' and focus on the science.

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doi:10.1038/nrclinonc.2016.113
Published online 26 Jul 2016

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Competing interests statement
The author declares no competing interests.

RETRACTION

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Nature Reviews Clinical Oncology http://dx.doi.org/10.1038/nrclinonc.2016.113 (2016)

I wish to retract the News & Views article 'Are circRNAs involved in cancer pathogenesis?' (*Nat. Rev. Clin. Oncol.* http://dx.doi.org/10.1038/nrclinonc.2016.113; 2016), owing to irreconcilable differences with the journal editors over correction of the text. Carlo M. Croce