Editorial

Circles in action, circles in function

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Circular RNAs were identified more than 20 years ago and thought, at the time, to simply represent rare biochemical curiosities, indicative of an "error-prone" splicing machinery (1-3). It was not until recently, with the advent of next generation sequencing, that such non-linear transcripts were found to be ubiquitously expressed and in certain cases being more abundant that the corresponding linear transcripts (4). Moreover, functional properties have started to be assigned to these molecules, including binding to proteins and acting as miRNAs traps (5,6). In their recent work Yu et al provide convincing evidence for the functionality of a circular RNA from the SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5 (*SMARCA5*) locus, cSMARCA5, containing exons 15 and 16 of the *SMARCA5* gene back-spliced together (7).

Using elegant and extensive analysis, the authors initially identified, from RNAseq data of five hepatocellular carcinoma (HCC) samples and the corresponding adjacent noncancerous liver (ANL), 4,142 circular RNAs originating from protein coding exons of 2,322 genes, 190 of which are differentially expressed in the HCC versus the ANL samples. Integrating expression data on the corresponding mRNAs revealed that about 30% of these circular RNAs are deregulated in the opposite direction compared to their linear transcripts in the HCC and ANL samples. This is indicative of functionality, since simple by-products of non-optimized splicing processes would be expected to accumulate in the same direction as the corresponding mRNA.

The authors focus on cSMARCA5, the circular RNA that is on top of their list of abundant circular RNAs expressed differentially in HCC/ANL and in the opposite direction than the corresponding mRNA. Its expression is reduced in HCC compared to ANL. Worth noting is that the abundance of this circular RNAs is, in fact, higher than the linear mRNA transcript (8), another indication of possible functionality. First they demonstrate that the presence of complementary, non-Alu sequences in the flanking introns promotes circularization of the SMARCA5 exons 15 and 16. Then that the DExH-Box Helicase 9 (DHX9), but not the additional RNA binding proteins adenosine deaminase 1 acting on RNA (ADAR1) and quaking (QKI), downregulates cSMARCA5 in HCC cell lines. Importantly, DHX9 expression is increased in HCC and negatively correlates to cSMARC5, providing a mechanistic rationalization to the reduced cSMARC5 expression. Also worth noting is that in mammary epithelial cells QK1 upregulates cSMARCA5 (9), again arguing that the production of this circular RNA is a result of context-specific regulatory events.

Subsequently, in a cohort of 163 human HCC and the corresponding ANL samples the authors demonstrate that low cSMARCA5 expression correlates with worse overall and recurrence-free survival. Biochemical analysis in HCC cell lines confirmed that modulating cSMARCA5 levels impacted on cell growth and migration, with low expression increasing and high expression decreasing these cellular outcomes. Moreover, subcutaneously or orthotopically implanted high cSMARCA5 expressing HCC cells reduced tumor growth compared to control cells. Similarly, *in vivo*

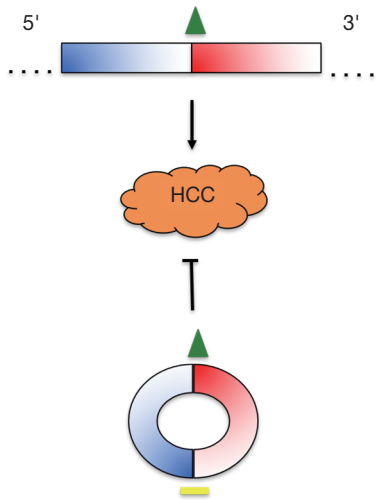

Figure 1 Schematic diagram on the impact of circular and linear SMARCA5 transcripts on HCC. The SMARCA5 mRNA promotes HCC, while the circular SMARCA5 RNA, composed of exons 15 and 16, inhibits HCC. Blue and red rectangles indicate the SMARCA5 exons 15 and 16, respectively. The position of the back-spliced junction of exon 16 to exon 15 in the circular RNA is indicated by a yellow bar. The position of the miR-17-3p and miR-181b-5p target sites overlapping the canonical splice junction of exon 15 to exon 16 is indicated by a green triangle. SMARCA5, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5; HCC, hepatocellular carcinoma.

injection of high cSMARCA5 expressing HCC cells in the tail vein of nude mice reduced lung metastasis relative to control cells. Thus, all data pinpoint to tumor suppressive properties of cSMARCA5 in HCC.

Circular RNAs and especially abundant circular RNAs are known to have the capacity to act as miRNA traps. The authors used standard miRNA prediction tools to identify potential miRNAs interacting with cSMARCA5 and selected 29, which are also expressed in HCC or ANL. Subsequently, they used the novel technique of circular RNA *in vivo* precipitation (circRIP) (10) to identify miRNAs from the 29-list that interact with cSMARCA5 in HCC cells. This analysis highlighted miR-17-3p and miR-181b-5p associating with cSMARCA5. Mutations of the two miRNA binding sites on cSMARCA5 followed by luciferase analysis confirmed these interactions, as also did the enrichment of cSMARCA5 by miR-17-3p and miR-181b-5p pull-down assays. Worth noting is that the two miRNA binding sites on cSMARCA5 are not positioned at the back-spliced junction of exon 16 to exon 15 but at the canonical splice junction of exon 15 to exon 16, in fact, the two binding sites partially overlap. This raises the possibility that linear SMARCA5 transcripts encompassing exon 15 and exon 16 could also be targeted by the two miRNAs.

miR-17-3p and miR-181b-5p are thought to promote HCC (11), consequently the authors focused on HCC tumor suppressors that can be targeted by the two miRNAs. They demonstrate that the tumor suppressor tissue inhibitor of metalloproteinase 3 (TIMP3), a target of both miRNAs, is highly regulated in HCC cells by changes in the expression of cSMARCA5. Consistent with a capacity of the circular RNA to trap miRNAs, high cSMARCA5 levels upregulate and low levels downregulate TIMP3. Moreover, and similar to cSMARCA5, TIMP3 expression was reduced in HCC, positively correlating with cSMARCA5 expression.

Thus, a mechanistic scenario consistent with a protective role of a circular RNA in HCC, which involves trapping miRNAs that downregulate a tumor suppressor, has been put forward.

It should be noted that in HCC, and in contrast to the reduced cSMARCA5 levels, the SMARCA5 mRNA (and the SMARCA5 protein) is increased relative to ANL. Moreover, the observed SMARCA5 mRNA and protein upregulation is thought to promote HCC (12). Given that the miRNA target sites identified on exons 15 and 16 are present not only in cSMARCA5 but also in the corresponding linear transcript, then the regulatory scenarios may, in fact, be even more complicated. MiR-17-3p and miR-181b-5p targeting of the SMARCA5 mRNA could potentially destabilize it inhibiting HCC. Additionally, trapping the miRNAs on the linear transcript may reduce their availability for TIMP3 downregulation, also potentially inhibiting HCC. Consequently, not only the levels of cSMARCA5 but also the relative expression of circular to linear SMARCA5 RNAs encompassing exons 15 and 16, may also impact on HCC development and prognosis (*Figure 1*).

Is cSMARC5 the only circular RNA that affects HCC? Well, the authors have produced a substantial list of circular RNAs, which are expressed at reasonable levels and differentially regulated in HCC versus ANL. Some may simply be passengers, but others may indeed influence disease development.

Is miRNA trapping the only mechanism of action of circular RNAs in HCC? Well, testing for miRNA interactions is an established experimental approach, while

the analysis of circular RNA interactions with proteins or other RNAs is not streamlined to the same extent, and consequently more difficult to address.

Taken together, the available evidence suggests that the potential of novel findings involving circular RNAs is, apparently, at hand and applying logical criteria for optimized selections, combined with technical innovations, will certainly pinpoint to additional functional implications of non-linear transcripts that are relevant for HCC and beyond. As a final note, the authors should also be commended that their long list of circular RNAs compiled through their extensive RNAseq analysis confirmed the existence of some of the earliest circular RNAs identified by RT/PCR more than 20 years ago (13).

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Footnote

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Ethical Statement: The author is 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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