Editorial

microRNA sponging by a new hearty circRNA

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The discovery of circular RNAs (circRNAs) as a widespread and abundant class of endogenous RNA in Eukarya (1-3) has sparked a previously unparalleled interest in this class of RNA molecules. CircRNAs, characterized by a covalently closed structure, are generated through back-splicing of one or more exons from protein-coding and non-coding genes and have been found in great number in diverse species such as human (1-3) and *Drosophila* (4). Almost in parallel with the realization of circRNAs as pervasive endogenous molecules, came the first functional characterization of circRNAs; two circRNAs, termed ciRS-7/CDR1as and *Sry* circRNA were found to bind to miRNAs and prevent these miRNAs from acting on their cognate targets (2,5). Since then, interest in assigning function to the thousands of other circRNAs that are expressed endogenously has expanded greatly, as reflected by the increasing number of publications on this subject. However, despite being subject to intense scrutiny, only a relatively small number of circRNAs has as of now been assigned a function and, for those that have, functions have been diverse: miRNA binding, transcriptional regulation, binding of RNA-binding proteins to either facilitate their interaction or sequester them as well as templates for translation (6-12). However, the ability of a circRNA to interact with and regulate miRNAs stand out as an especially intriguing function seeing as the covalently closed structure provides these circRNAs with a unique form of protection from miRNA-mediated exonucleolytic decay (2,5,13). Hence, miRNA-sponging is to date one of the only functions discovered where the circular structure provides a unique advantage compared to linear RNA. However, bioinformatics analysis of circRNAs has disclosed that only a small fraction of circRNAs possess a substantial number of binding sites for a specific miRNA (13,14). This finding seems to indicate that miRNA sponging may not be a general function of this class of RNA (14). However, despite this initial discouraging analysis, the number of circRNAs proposed to function by direct interaction with miRNAs continue to rise, as listed elsewhere (15,16). In general, these newly characterized miRNA "sponges" possess a relatively small number of canonical miRNA sites, which appears to be enough to mediate the regulation. The recent paper "Circular RNA mediated cardiomyocyte death via miRNA-dependent upregulation of MTP18 expression" by Wang *et al.* adds yet another voice to this ensemble of circRNAs proposed to act as miRNA sponges.

In their paper (17), Wang *et al.* disclose that in cardiomyocytes the circRNA termed MFACR interacts with miR-652-3p. In turn, the interaction between MFACR and miR-652-3p prevents this miRNA from acting on its cognate target, MTP18, which it inhibits translation of.

To establish and validate this set of interactions between MFACR - miR-652-3p and MTP18 - miR-652-3p Wang and colleagues relied both on bioinformatics predictions of miR-652-3p sites in MFACR and MTP18 as well as on experimental validation. With regard to the bioinformatics analysis, the tool RNA-hybrid was used to search for miRNA binding sites, which resulted in the finding of one miR-652-3p binding site in the 3'UTR of MTP18 mRNA and fifteen binding sites in MFACR. However, this analysis seems to have been carried out with very relaxed parameters, as only two of the fifteen miRNA target sites predicted in MFACR can be described as conventional upon manual inspection, illustrating that many of the fifteen predicted miRNA target sites can be considered atypical. Although it is not

impossible to imagine such sites being used, extra caution should be taken and rigorous biochemical validation is necessary before such predicted sites can be considered relevant. In the case of miR-652-3p and its interaction with MFACR, Wang and colleagues experimentally confirmed the proposed interaction by showing that they could capture MFACR both using a biotinylated miR-652-3p and using AGO2 immunoprecipitation in primary cardiomyocytes. Conversely, miR-652-3p could be pulled down with a probe designed against MFACR. Hence, this experimental validation suggests that MFACR is able to interact with miR-62-3p, but whether all of the fifteen predicted sites are used remains unknown. With regard to the interaction between MTP18 mRNA and miR-652-3p, the sole target site identified using RNA-hybrid is not captured by other tools such as TargetScan when standard setting are used (18). However, the interaction was validated by showing decreased regulatory effect of miR-652-3p on MTP18 levels upon transfection of antagomirs or a target blocker into primary cardiomyocytes. Furthermore, mutation of the identified miR-652-3p binding site, rendered MTP18 resistant towards a miR-652-3p mimic, as tested using both luciferase assays and overexpression of MTP18 constructs with or without the mutation in primary cardiomyocytes.

With a successful validation of the interactions between MFACR and miR-652-3p as well as between miR-652-3p and MTP18 mRNA, Wang *et al.* set out to determine the function of this uncovered MFACR-miR-652-3p-MTP18 pathway. As MTP18 is a membrane protein found on mitochondria that is required for mitochondrial fission (19) and in light of the recent association between cardiac diseases and abnormal mitochondrial fission and fusion (20,21), Wang *et al.*, investigated the role of the MFACR, miR-652-3p-MTP18 axis in myocardial infarction. For this purpose, Wang *et al.* applied two model systems to mimic myocardial infarction, a situation characterized by oxygen deprivation followed by cardiomyocyte apoptosis. The *in vitro* model system involved cultured primary cardiomyocytes that were subjected to complete oxygen deprivation followed by re-oxygenation. The *in vivo* system comprised mice with a snare surgically inserted around a blood vessel. To mimic myocardial infarction, the snare was tied for forty-five minutes, thereby inducing ischemia, followed by three hours of reperfusion.

In both the *in vitro* and *in vivo* mouse models, lack of oxygen lead to an increase in MTP18, a decrease in miR-652-3p and an increase in MFACR level. Furthermore, oxygen deprivation induced fragmentation of mitochondria as well as cardiomyocyte apoptosis. Additionally, in the *in vivo* model, removal of oxygen lead to an infarct as well as cardiac dysfunctions. This phenotype could be partially rescued by re-directing the expression of each of the three components in the MFACR-miR-652-3p-MTP18 pathway towards that of the non-oxygen deprived condition using (I) siRNA-mediated depletion of MTP18 or (II) overexpression of miR-652-3p with a mimic or (III) by siRNA-mediated depletion of MFACR. To substantiate that the action of MFACR depends on miR-652-3p, Wang and colleagues conducted a co-depletion assay, where both MFACR and miR-652-3p was depleted, by a siRNA and an antagomir respectively. This co-depletion reversed the reduced *MTP18* protein level, mitochondrial fragmentation and cellular apoptosis seen when only MFACR was depleted, suggesting that the effect elicited by MFACR during oxygen deprivation is in fact carried out by miR-652-3p. In turn, that miR-652-3p is the agent directly responsible for lowering the MTP18 levels and thereby lowering mitochondrial fission and apoptosis, was demonstrated using a target protector for MTP18. In the presence of this target protector, miR-652-3p was unable to inhibit mitochondrial fission and apoptosis, illustrating that MTP18 is a direct target of miR-652-3p.

Hence, the study performed by Wang *et al.* provides new interesting insights into the role of a circRNA in pathophysiological conditions in the heart. Although other papers have described the involvement of circRNAs in physiological and pathophysiological functions in the heart both through interaction with miRNAs or proteins (8,22,23), the pathway uncovered by Wang *et al.* has not been described before. However, as also acknowledged by the authors, the mechanism by which the uncovered MFACR–miR-652-3p–MTP18 pathway is activated during oxygen deprivation remains undetermined and further investigations are required in order for this pathway to be fully elucidated. Nonetheless, with this paper Wang *et al.* contributes to the characterization of the class of circRNA by adding the label "miRNA regulator" to MFACR. Although other functions have been accredited to circRNAs, by far the most frequently dispensed one today is that of the miRNA sponge. As the vast majority of circRNAs still awaits functional characterization, the question is whether this "miRNA regulator" label will be applied to many more circRNAs or whether research so far has been skewed towards identification of functions for which there

is an established methodological approach, limiting our characterization of circRNAs to a small subclass. Maybe the most astonishing observation in this paper was that either direct intravenous injections of naked miR-652-3p mimic or delivery of adenovirus expressing a small interfering RNA towards MTP18 or MFACR circRNA in all three cases almost halved the number of apoptotic cells and the myocardial infarction area after ischemia/reperfusion injury. This result may path the way for new therapeutic strategies for treatment and management of heart diseases.

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