International Journal of Cardiology 290 (2019) 134–137

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Short communication

circRNA-miRNA cross-talk in the transition from paroxysmal to permanent atrial fibrillation

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ARTICLE INFO

Article history:
Received 22 March 2019
Accepted 23 April 2019
Available online 8 May 2019

Keywords:
Atrial fibrillation
Circular RNAs
Micro-RNAs
Regulatory networks
RNAseq

ABSTRACT

Background: Atrial fibrillation (AF) is the most prevalent cardiac arrhythmia in western countries. The factors governing the progression of AF to a permanent chronic condition are still not well characterized. Among epigenetic factors, non-coding RNAs (ncRNAs) such as miRNAs and lncRNAs have been recently described as important players involved in the AF progression. We hypothesize about the existence of additional regulatory layers in AF involving an intricate cross-talk between different ncRNA species, namely miRNAs and circRNAs for the establishment of a chronic AF condition.
Methods and results: We have performed an unbiased study analyzing the expression profile for miRNAs and circRNAs in left-atrial biopsies from patients with paroxysmal and permanent AF by RNA-seq. The transition from paroxysmal to permanent AF is characterized by a pattern of down-regulated miRNAs, concomitant to the appearance of specific circRNA species. The analysis of the sponging activities of the circRNAs exclusively expressed in permanent AF samples, allowed us to determine that they could be responsible for the downregulation of specific miRNAs in establishment of a permanent AF condition.
Conclusion: Sponging activity of circRNAs sequestering specific miRNAs is an important factor to be considered for the determination of the molecular mechanisms involved in AF progression.

1. Introduction

Atrial fibrillation (AF) is the most prevalent form of cardiac arrhythmia in western countries responsible for an increased risk of embolic stroke and heart failure and being a cause of morbidity and mortality especially of elderly individuals [1]. After a trigger event that starts the disease, the clinical manifestations of AF often include the transition from a paroxysmal AF (where arrhythmic crises are sporadic and reversible) to a long-lasting and chronic form of the condition (permanent AF) [2]. Despite of the existence of some genetic factors conditioning the AF progression, the molecular players involved in this transition are still unknown [3].

Cardiac tissue requires a delicate control of its functions at the molecular level which will ensure a mechanical, electrical and chemical synchronization. In the non-proliferative myocardium, some epigenetic factors such as non-coding RNAs (ncRNAs) have been characterized as important players involved in its function but also in pathological conditions as AF [4,5]. The role of post-transcriptional ncRNA regulators in specific events related with AF has started to be unveiled in the last years with the characterization of specific miRNAs involved in atrial remodeling [6], cardiomyocyte apoptosis [7], valvular pathologies [8] and disease progression [9]. Other ncRNAs such as long ncRNAs (lncRNAs) have been also described as important players in AF either in patients [5] or in animal models [10]. Interestingly, non-coding transcriptome is composed by several molecular species that have entangled relationships based on molecular interactions. In this context, circular RNAs (circRNAs) are a specific family of ncRNAs generated by non-canonical splicing of coding genes, and are able to interact with other ncRNAs, namely miRNAs, sequestering them by a sequence-driven sponging effect [11]. Despite the already existing evidences pointing to the importance of circRNAs in AF, their roles in the disease progression are still not understood [12].

In a previous work, we characterized the miRNA expression profile in atrial tissue from paroxysmal and permanent AF patients, showing a strong down-regulation of miRNAs related with disease progression [9]. We hypothesize about the existence of a sponging mechanism driven by circRNAs over miRNAs in the transition from paroxysmal to

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https://doi.org/10.1016/j.ijcard.2019.04.072
0167-5273/

permanent AF that could explain the down-regulation pattern of miRNAs. The existence of this regulatory cross-talk between ncRNA species would be an important landmark for understanding the molecular mechanisms involved in AF progression.

2. Methods

Left atrial tissue samples collected during surgical valvuloplasty were used to characterize the coding and non-coding transcriptome expression profile from a cohort of 14 patients: 3 showing permanent AF, 5 with paroxysmal AF and 6 controls in sinus rhythm. The study was approved by the Ethical Committee of the Santa Maria Hospital and the Faculty of Medicine of the University of Lisbon, Portugal, in agreement with the ethical guidelines of the 1975 Declaration of Helsinki, and all the enrolled patients provided written informed consent.

Biopsies were mechanically homogenized with a disperser and high-quality total RNA was isolated from the samples by using the Qiagen RNAeasy mini-kit, and fractionated by size prior to Illumina® library preparation for high-throughput sequencing. Libraries for small RNAs (<200 nt) and large RNAs were separately prepared and sequenced at the GeneCore facility, EMBL, Heidelberg, Germany. Sequence reads from large RNAs were aligned with the human genome (UCSC assembly hg38) using Bowtie2 software [13], and the unaligned reads used for circRNA characterization by the Uroborus circRNA identification tool [14]. For miRNA characterization, small RNA reads were aligned to miRBase 22.1 using Oasis2 software [15].

Differentially expressed circRNAs and miRNAs in paroxysmal and permanent AF were determined by comparison with a group of patients in sinus rhythm by using the Cufflinks suite [16] and the Oasis2 [15] software respectively, considering as significantly differentially expressed those species with adjusted p-value < 0.05 and $-1.0 > -\log2(\text{Fold change}) > 1.0$. Functional sponging activity of circRNAs over miRNAs was analyzed by the prediction of miRNA target binding sites over the sequences of selected circRNAs using the miRanda software in its standalone version [17]. Regulatory networks between circRNAs and miRNAs in AF were represented in the NAViGaTOR suite [18].

3. Results

In order to analyze the functional regulatory networks established between miRNAs and circRNAs during AF progression, we quantified the expression levels of these families of ncRNAs by RNAseq in a selected group of atrial biopsies from a cohort of 14 patients: 3 showing permanent AF, 5 with paroxysmal AF and 6 controls in sinus rhythm. Differential expressed miRNAs in paroxysmal and permanent AF samples in reference to the sinus rhythm group showed a striking pattern of down-regulated miRNAs in permanent AF samples, composed by 112 miRNAs (Fig. 1A, Suppl. Table 1). Concomitant to the miRNA down-regulation, an increase in the detected circRNA species in the transition from paroxysmal to permanent AF was observed (Fig. 1B). 40 circRNAs were exclusively detected in permanent AF samples, being generated by the non-canonical splicing of 18 coding genes: SNRNP200 PLCG1, CDH11, FBLN2, COL14A1, TIMP3, COL3A1, MYH7, LRP6, TPM1, RYR2, MLIP, PAM, RBM27, FBN1, GAN, PDZD2, and PLEC. Among the exclusively expressed circRNAs in permanent AF samples, only 9 of them were already functionally characterized and annotated in circBase [19]: hsa_circ_0025470 (LRP6 generated); hsa_circ_0035132 and hsa_circ_0035148 (FBN1 generated); hsa_circ_0057344 (COL3A1 generated); hsa_circ_0085900 (PLEC generated); hsa_circ_0105720 (CDH11 generated); hsa_circ_0112651, hsa_circ_0112664 and hsa_circ_0112682 (RYR2 generated) (Suppl. Table 2).

The inversely correlated expression pattern between circRNAs and miRNAs in permanent AF samples suggested the presence of a regulatory cross-talk network based on the sponging activity of circRNAs over miRNAs (Fig. 1C). Taking into consideration only the 9 circRNAs exclusively expressed in permanent AF samples and annotated in circBase, our analysis determined that 95% of the down-regulated miRNAs (107 of 112 miRNAs) have at least one functional target in any of these circRNAs (Fig. 2A).

MiRNA target multiplicity within circRNAs increase the sponging capacity of these molecular species. The analysis of the number of targets for down-regulated miRNAs in permanent AF samples for specific circRNAs predicts that the sponging activity would be more relevant for some miRNAs (Fig. 2B). This is the case of hsa-miR-181d-5p, hsa-miR-3180-3p, hsa-miR-6868-3p and hsa-miR-2277-5p, which showed multiple repetitive targets in the sequence of the analyzed circRNAs.

4. Discussion

Pervasive transcription of the human genome generates thousands of regulatory ncRNAs that are involved in cell homeostasis and disease [10]. Regulatory effect of ncRNAs is exerted mainly by controlling the genetic flow from coding genes to proteins, but recent evidences

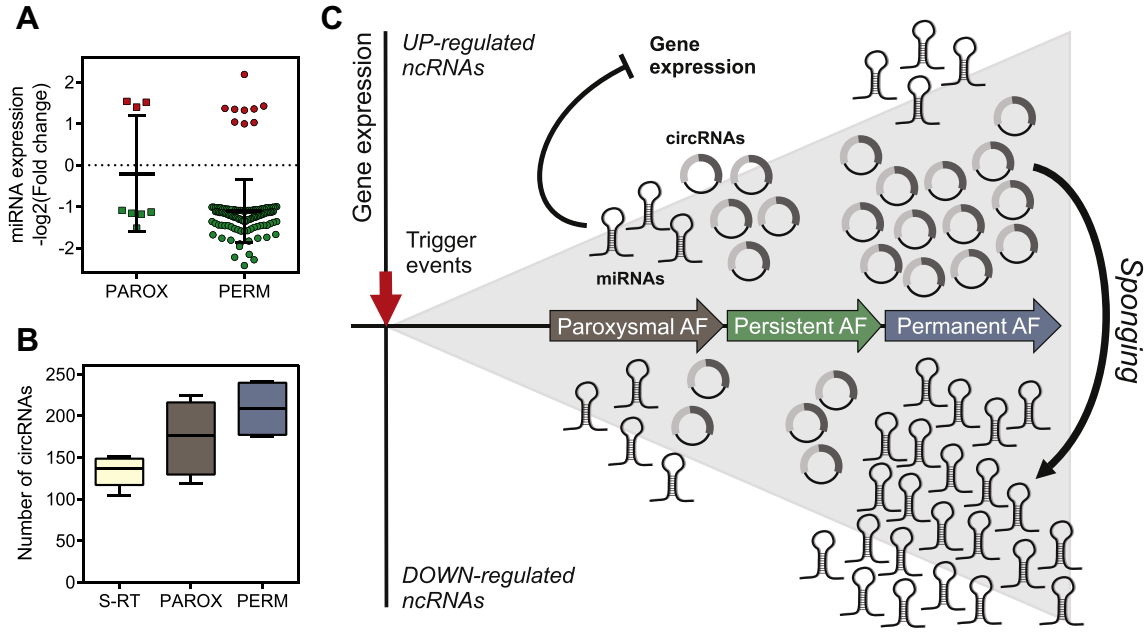

Fig. 1. The miRNA and circRNAs expression landscape in atrial appendages from patients with paroxysmal and permanent AF. A, differentially expressed miRNAs as detected by small RNAseq in paroxysmal (PAROX) and permanent (PERM) group of samples compared with the sinusal rhythm control group. B, number of circRNAs detected by RNAseq in each group of samples (S-RT: sinusal rhythm control group, PAROX: paroxysmal group and PERM: permanent group), depicted as a box and whiskers graph (5–95 percentile). C, overall hypothesis and working model of our study, whereas the transition from paroxysmal to permanent AF would be characterized by an increase of the expression of specific circRNAs able to sponge miRNAs, and producing a decrease in the expression levels of these post-transcriptional regulators.

showed that ncRNA species are also able to interact to fine tune their effects. The regulatory action ncRNAs is especially important in organs such as the heart where a delicate cellular synchronization is needed [4]. We previously demonstrated that miRNAs could be considered as important molecular players in the transition from paroxysmal to permanent AF characterized by an important down-regulation of the miRNA expression levels (Fig. 1A, Suppl. Table 1) [9]. A deeper analysis of the non-coding transcriptome of AF patients in different stages showed that the down-regulation of miRNA expression in permanent AF is also accompanied by the existence of a group of specific circRNAs

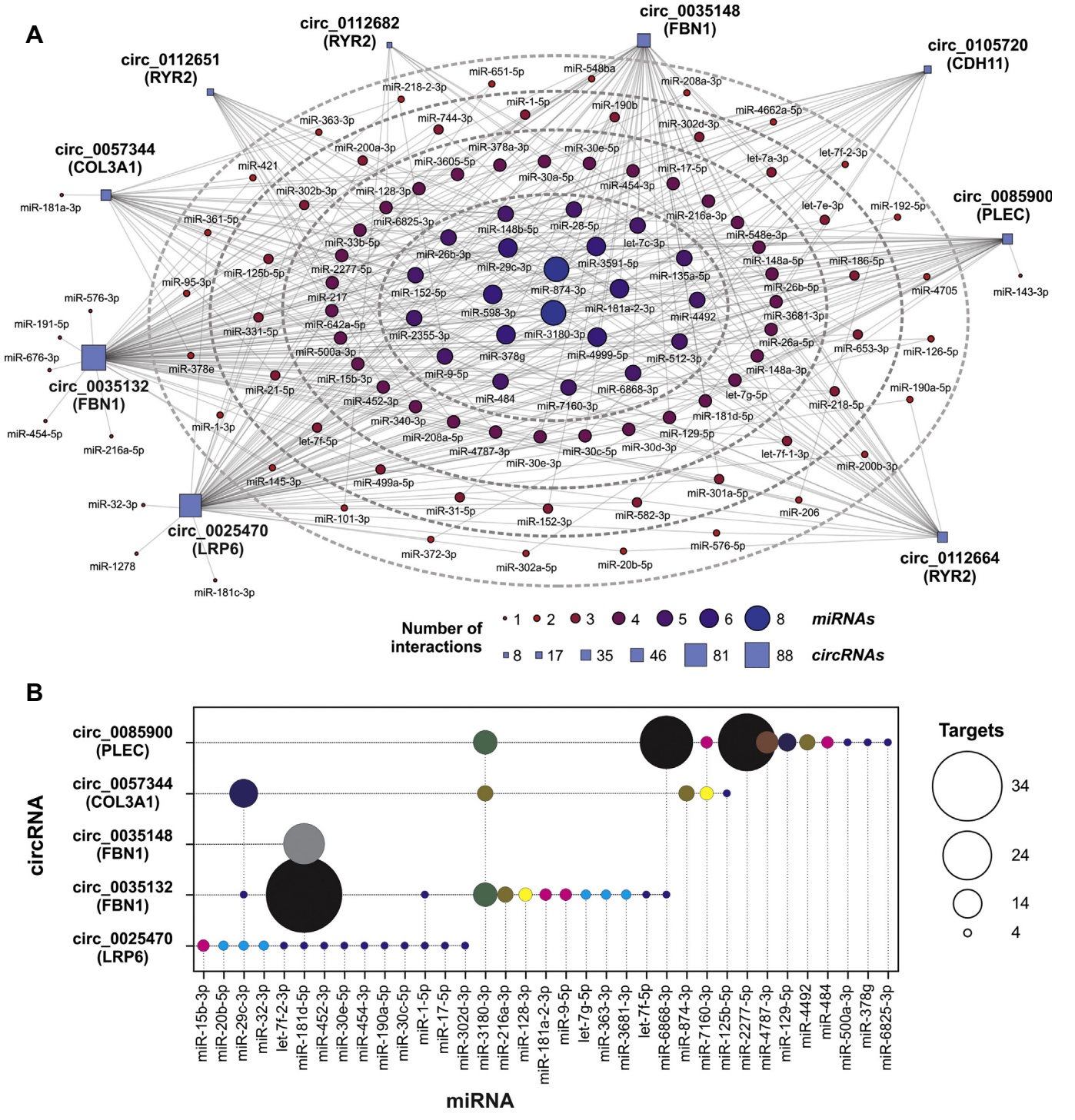

Fig. 2. miRNA-circRNA regulatory interactions in permanent AF characterized in circRNAs exclusively expressed in permanent AF samples and annotated in circBase. The depicted interactions are based on the predicted sponging activity of circRNAs over down-regulated miRNAs in permanent AF samples. A, overall circRNA-miRNA crosstalk by circRNAs exclusively expressed in permanent AF samples and already annotated in circBase and down-regulated miRNAs in the same group of patients, prepared with NAViGaTOR software [18]. CircRNAs are depicted as squares and miRNAs as circles, with sizes proportional to the number of functional relationships established. Each connecting line represents the presence of at least one miRNA target in a particular circRNAs. Dashed ellipsoids were traced to show the different number of interactions levels between miRNAs and circRNAs. B, sponging capabilities of hsa_circ_0085900, hsa_circ_0057344, hsa_circ_0035148, hsa_circ_0035132 and hsa_circ_0025470, quantified as the presence of >3 targets for particular miRNA in each circRNAs. Diameters of circles are proportional to the number of miRNA targets in each circRNAs, as indicated in the figure legend. In both panels, the canonical nomenclature of circRNAs and miRNAs has been simplified for representation purposes by eliminating the "hsa" prefix. The source coding gene for each circRNAs is indicated in brackets after the circRNA name.

which are non-detectable in other phases of the disease (Fig. 1B, Suppl. Table 2), which could be compatible with a regulatory circRNA-miRNA crosstalk network.

Detection and characterization of circRNAs is strongly dependent on the quality of the sequencing data but also on the algorithm used for its analysis [14]. For this reason we tested our working hypothesis (Fig. 1C) by using only the circRNAs exclusively expressed in permanent AF and already annotated in circBase [19], however the existence of new circRNAs species could also contribute to the overall disease phenotype [12]. Our results showed that >95% of the down-regulated miRNAs in permanent AF could be potentially sponged by the exclusively expressed circRNAs in the same group of samples, and clearly support the existence of a circRNA-miRNA functional crosstalk in permanent AF patients, that could be an important factor for disease progression (Fig. 2A). The analysis of the relative sponging activities of the selected circRNAs, characterized hsa-miR-181d-5p, hsa-miR-3180-3p, hsa-miR-6868-3p and hsa-miR-2277-5p as important targets to be considered. Despite the lack of knowledge about the majority of these miRNAs in the context of AF, the role of hsa-miR-181 family in AF has been previously characterized [20], and also supports the involvement of circRNAs in disease progression.

The main limitation of this study relies on the validation and characterization of this ncRNA cross-talk mechanism in proper experimental models and patients. Selection of animal models for controlled environment studies is still limited by the differences in circRNAs generation in humans and animals. On the other hand, cellular models based on the culture of primary cardiac cells are also limited by their difficulty to setup and to manipulate, even if they are obtained from induced pluripotent stem cells (iPSCs). Our study has been based on experimental data obtained from patients, but we also acknowledge the limitations of the prospective analysis based on an in silico approach to determine the functional relationships between circRNAs and miRNAs. Considering all these facts, we still believe that this study opens a new avenue for understanding the molecular players potentially involved in the complex mechanisms subjacent to AF progression with the prospective to be applied in patient treatment and disease prognosis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.04.072.

Acknowledgement of grant support

The present research work has been performed with the support of the Fundação para a Ciência e a Tecnologia (FCT), research grant PTDC-MED-GEN-29389-2017.

Conflict of interest

The authors declare no potential conflict of interest.

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