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Functional Role and Mechanism of microRNA-28b in Atrial Myocyte in a Persistent Atrial Fibrillation Rat Model

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Background: Persistent atrial fibrillation has been indicated to be related with microRNA-28b. However, the exact role of microRNA-28b in persistent atrial fibrillation needs to be further elucidated. Therefore, this study aimed to establish a rat model of persistent atrial fibrillation to investigate the level of microRNA-28b in atrial myocytes and to explore the molecular mechanism involved.





Material/Methods: A persistent atrial fibrillation model was established in rats by using chronic rapid atrial pacing induction. The size of the heart was measured by ultrasonic method. The expression of microRNA-28b in left atrial myocytes was quantified by RT-PCR. Cardiomyocytes were isolated and cultured to detect cell proliferation and apoptosis by MTT and flow cytometry, respectively. The specific inhibitor of ERK signaling pathway, PD98059, was used to further illustrate the role of ERK signaling pathway in the modulation of cardiomyocytes in persistent atrial fibrillation.

Results: MicroRNA-28b was up-regulated in the experimental rat model with persistent atrial fibrillation. The proliferation of cardiomyocytes was significantly inhibited with potentiated apoptosis. Blockage of the ERK pathway suppressed the microRNA-28b expression and inhibited cell apoptosis.

Conclusions: microRNA-28b-induced growth inhibition and cell apoptosis of atrial myocytes was observed in the rat model with persistent atrial fibrillation, via activation of the ERK signaling pathway.

MeSH Keywords: **Atrial Natriuretic Factor • Cellulose 1,4-beta-Cellobiosidase • Central Nervous System Vascular Malformations • Mitogen-Activated Protein Kinase 3**

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Background

With the transition of life styles, the incidence of cardiovascular diseases, including persistent atrial fibrillation, is continuously increasing [1]. Persistent atrial fibrillation severely affects quality of life [2]. It is defined as continuous atrial fibrillation that lasts more than 7 days [2,3]. The classical treatment approaches, including medication and surgery, are important in the treatment of persistent atrial fibrillation [4], but also may cause adverse effects, including hemorrhage [5]. Therefore, it is necessary to develop novel therapies that have higher efficacy [6–8].

As a small-molecule RNA, microRNA has a length of between 17 and 23 bp [9,10]. Although not coding proteins, microRNA has pluripotent biological functions, including modulation of cell growth and survival [11], as well as regulating cell proliferation, cell cycle [12], autophagy, and the signaling pathway [13].

microRNAs can improve the therapy of heart failure patients [14] and play an important role in cardiac resynchronization therapy [15]. Furthermore, the microRNA expression has been demonstrated to be altered after atrial fibrillation catheter ablation and could be used as atrial fibrillation fibrotic and electrical alterations biomarkers [16]. A recent study has suggested a correlation between microRNA-28b and persistent atrial fibrillation [17], although its exact function in the structural reconstruction of cardiomyocytes needs further investigation [8]. microRNA-28b has been implicated in modulating cell growth and survival [19]; therefore, this study investigated the potential role of microRNA-28b in cardiomyocytes of rats with persistent atrial fibrillation.

The ERK signaling pathway is mediated by certain serine/threonine kinases [20]; it is widely distributed [21] and mediates cell growth and survival [22], cell proliferation and cell cycle [23], autophagy and signaling pathway [24]. Recent studies showed activation of the ERK pathway in cardiomyocytes during persistent atrial fibrillation in mammals and humans [25,26]. Therefore, dysregulation of cell growth and survival of cardiomyocytes may be a cause of persistent atrial fibrillation [27].

In this study, we generated a rat model of persistent atrial fibrillation, in which the role of microRNA-28b and the ERK signaling pathway in cardiomyocytes were analyzed in conjunction with signaling pathway analysis.

Material and Methods

Animal model

A total of 192 male SD rats (Shandong University, China) were prepared for establishment of a persistent atrial fibrillation

model using a chronic rapid atrial pace marker, as previously documented [27]. In brief, a circular pacemaker electrode was fixed on the left superior pulmonary vein. Another recording electrode was fixed on the left atrial wall. One pulse stimulator (1600 per min) was connected to the pacemaker electrode. The stimulus was applied every 2 days, in parallel with electrophysiological recordings. The successful generation of the model was achieved when the duration of persistent atrial fibrillation exceeded 12 h. A sham group was also recruited with electrode implantation but no stimulus.

The establishment of the AF model was defined as a rapid (>800 beats/min) irregular atrial rhythm, and AF inducibility was defined as AF lasting for at least 1 s immediately after the 6-burst cycle protocol. If AF was induced after <6 burst pacing cycles, burst pacing was suspended to avoid interfering with the evolution of AF. AF duration was determined in each rat as the mean duration of all AF episodes.

This study was approved by the Experimental Animal Ethics Committee of Qilu Hospital of Shandong University.

Ultrasonic examination

Using methods previously described [22], we measured all parameters of echocardiography, including ejection fraction, diameter of outflow tract in the right ventricle, and inner diameters of left/right ventricles and atrium.

Isolation and culture of cardiomyocytes

Using previously reported methods [28], we isolated and cultured cardiomyocytes from persistent atrial fibrillation and sham rats. In brief, heart tissues were first digested with 0.1% trypsin and re-suspended in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS). A specific cell-sorting column containing specific antibody against cardiomyocyte markers troponin I and troponin T was used to purify cells for continuous incubation in a humidified chamber at 37°C for 48 h.

MTT assay

ERK signaling pathway inhibitor PD98059 (100 nM) was used to incubate isolated cardiomyocytes for 12 h. After changing the medium at 24 h, MTT assay was performed to detect the growth and survival of cardiomyocytes, as documented previously [29].

Cell apoptosis assay

Cultured cardiomyocytes from the experimental and sham groups were examined for apoptosis using flow cytometry by detecting plasma membrane translocation of phosphatidylserine

Table 1. Echocardiography of rat hearts. The *t* test was used to compare means across groups. All of the P values were greater than 0.05, and illustrated no significant differences.

Group	Sham (n=96)	Experiment (n=96)	t value	P value
LVEF (%)	57.20±4.30	56.78±4.22	0.356	0.723
LVIDd (mm)	50.75±3.86	50.14±4.11	0.545	0.588
LVIDs (mm)	27.55±2.35	27.36±2.00	0.318	0.752

LVEF – left ventricle ejection fraction; LVIDd – left ventricle internal diameter at end-diastole; LVIDs – left ventricle internal end systolic diameter.

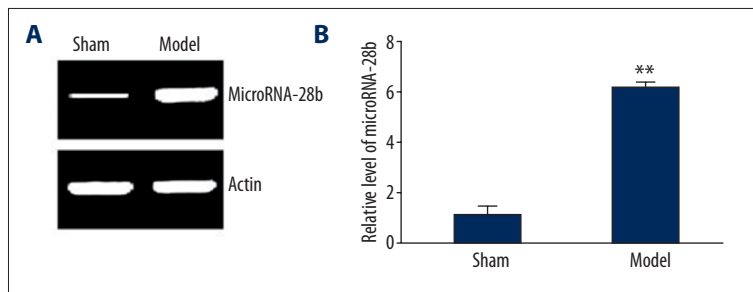


Figure 1. (A, B) MicroRNA-28b level in left ventricular cardiomyocytes. One-way analysis of variance (ANOVA) was used to compare means across groups. ** $P < 0.01$ represents the comparison of levels of microRNA-28b in the model group vs. the sham group.

with FITC-Annexin-V dye (Beyotime, China), as previously reported [30]. Caspase-3 activity in cardiomyocytes was measured by test kits (Sigma, USA), as reported previously [31].

RT-PCR

Total RNA was extracted from cardiomyocytes of all groups using a previously documented method [32]. RT-PCR was then performed to quantify the level of microRNA-28b using actin as the internal reference. Primers used included: microRNA-28b-forward, 5'-CTCAG AGAGT TTGAT-3'; microRNA-28b-reverse, 5'-AGAGT TTGAT CATGG-3'; actin-forward, 5'-TTAG ACGTT AGAGT-3'; actin-reverse, 5'-TGATA TGAGT CTGAG-3'.

Western blotting

Activation of the ERK signaling pathway in experimental and sham rats was tested by Western blot analysis [33]. In brief, all cells were lysed to extract total proteins, which were then separated by SDS-PAGE and transferred onto a PVDF membrane. Antibody against ERK and p-ERK (Beyotime, China) were used to detect protein levels.

Statistical analysis

All data were processed in SPSS 13.0 software, and are presented as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across groups. Statistical significance was defined at $p < 0.05$.

Results

Echocardiography

Ultrasonic examination of all rats with induced persistent atrial fibrillation showed no significant structural abnormalities as compared to sham rats. Quantitative parameters, including ejection fraction, inner diameter of right ventricle efflux tract, and inner diameters of left/right ventricle and atrium, all had no significant difference between the 2 groups (Table 1), suggesting no systemic heart disease in model rats.

MicroRNA-28b expression level

As shown in Figure 1, microRNA-28b expression level in cardiomyocytes in rats with persistent atrial fibrillation was significantly elevated compared to that in the sham group ($P < 0.05$).

Growth inhibition of rat cardiomyocytes

As shown in Figure 2, MTT assay showed significantly decreased growth of cardiomyocytes in rats with persistent atrial fibrillation as compared to those in the sham group ($P < 0.01$).

Apoptosis of cardiomyocytes

The percentage of apoptotic cells, as shown by the membrane translocation of phosphatidylserine, was significantly higher in the persistent atrial fibrillation group when compared to the sham group ($P < 0.05$, Figure 3). Moreover, the activity of caspase-3 in model rats was also increased ($P < 0.01$, Figure 4).

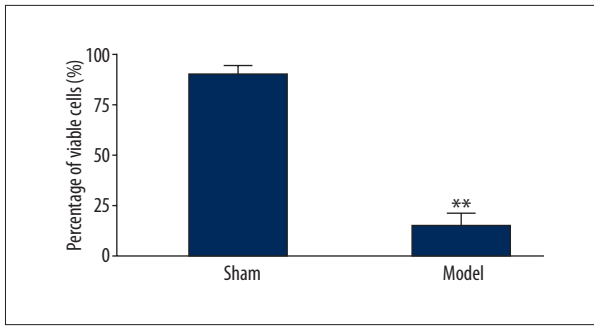


Figure 2. Growth of cardiomyocytes. One-way analysis of variance (ANOVA) was used to compare means across groups ** $P < 0.01$ represents the comparison of percentage of viable cells in the model group vs. the sham group.

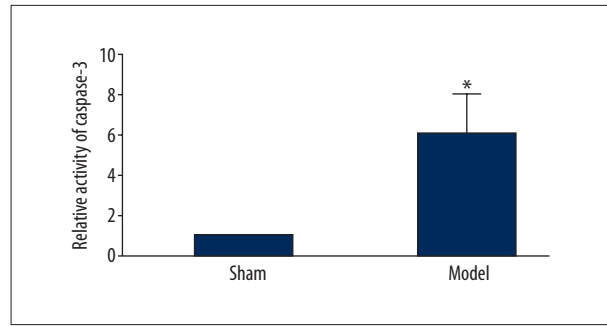


Figure 4. Caspase-3 activity and apoptosis in cardiomyocytes. One-way analysis of variance (ANOVA) was used to compare means across groups. * $P < 0.05$ represents the comparison of activity of caspase-3 in the model group vs. the sham group.

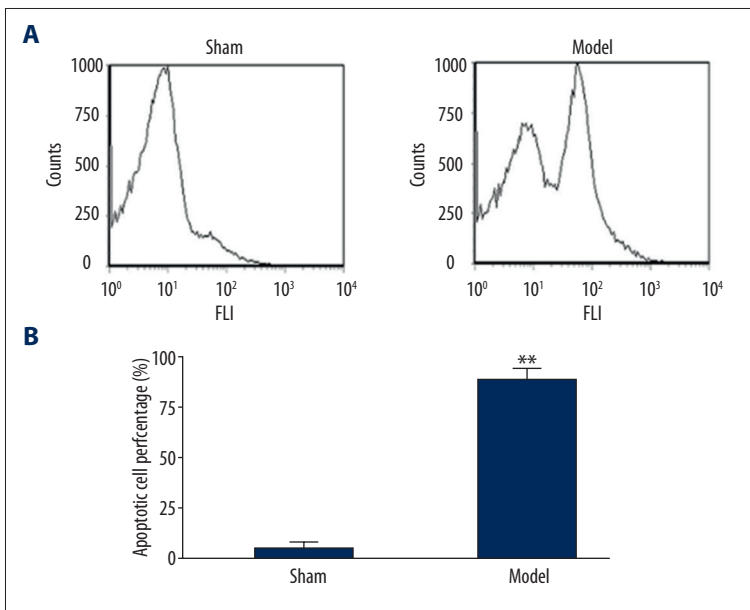


Figure 3. (A, B) Apoptosis of cardiomyocytes. One-way analysis of variance (ANOVA) was used to compare means across groups ** $P < 0.01$ represents the comparison of the apoptosis cell percentage in the model group vs. the sham group.

These results suggest the occurrence of apoptosis in cardiomyocytes in the model.

ERK activation in persistent atrial fibrillation rats

As shown by Western blot analysis, the phosphorylated form of ERK in cardiomyocytes from model rats was significantly increased (Figure 5), suggesting the activation of the ERK pathway in persistent atrial fibrillation.

Blocking of ERK activation suppressed cell apoptosis

We further used a specific blocker of the ERK signaling pathway (PD98059, at 100 nM) to treat cultured cardiomyocytes for 12 h. Results showed significantly decreased apoptosis of cardiomyocytes after treatment with inhibitor (Figure 6), suggesting

participation of the ERK signaling pathway in the apoptosis of cardiomyocytes in rats with persistent atrial fibrillation.

Discussion

Persistent atrial fibrillation severely affects patient quality of life [2]. A recent study indicated the involvement of microRNA-28b in persistent atrial fibrillation [7], but the role of microRNA-28b in pathogenesis requires further investigation. This study aimed to investigate the correlation between microRNA-28b and disease pathogenesis, in addition to exploring the molecular mechanisms.

We showed elevated expression of microRNA-28b in cardiomyocytes from rats with persistent atrial fibrillation, indicating the potential involvement of microRNA-28b in disease

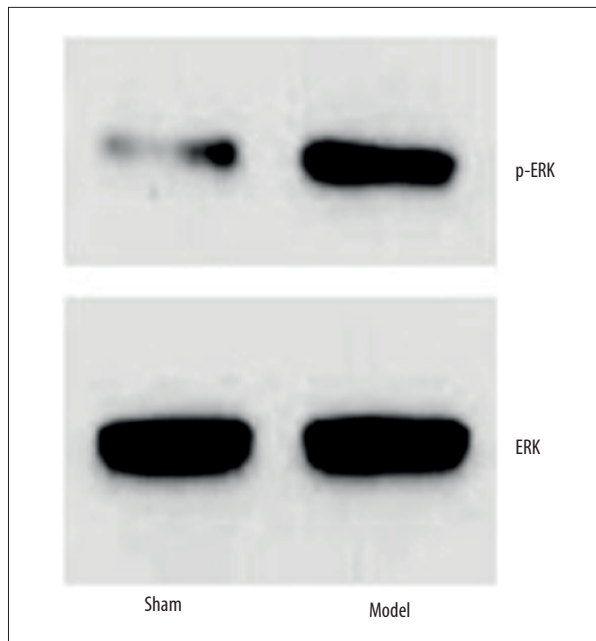


Figure 5. ERK activation in cardiomyocytes. The p-ERK and ERK expression were examined by Western blot.

pathogenesis. Furthermore, the MTT assay showed inhibited cell growth in cultured cardiomyocytes isolated from model rats, but remarkable apoptosis of cardiomyocytes. Activation of the ERK signaling pathway and consequently elevated cell

apoptosis suggested the suppression of cardiomyocyte growth and enhanced apoptosis by microRNA-28b via activating the ERK signaling pathway. Certain limitations existed in this study. First, no clinical samples from persistent atrial fibrillation patients were collected to examine microRNA-28b level, ERK activation, and cardiomyocytes apoptosis. Second, we did not treat model rats or patients to interfere with the microRNA-28b level to observe possible improvement. Third, exogenously transfected microRNA-28b has the inherent problem of lower efficacy, making the determination of the correlation between microRNA-28b and disease difficult. Future studies thus should focus on the RNA interference approach to validate the correlation between microRNA-28b and persistent atrial fibrillation.

Conclusions

Our innovative experimental results show microRNA-28b induces growth inhibition and cell apoptosis of atrial myocytes in a rat model of persistent atrial fibrillation via activating the ERK signaling pathway, suggesting that microRNA-28b might be a novel target in the treatment of persistent atrial fibrillation.

Disclosure of conflict of interest

None.

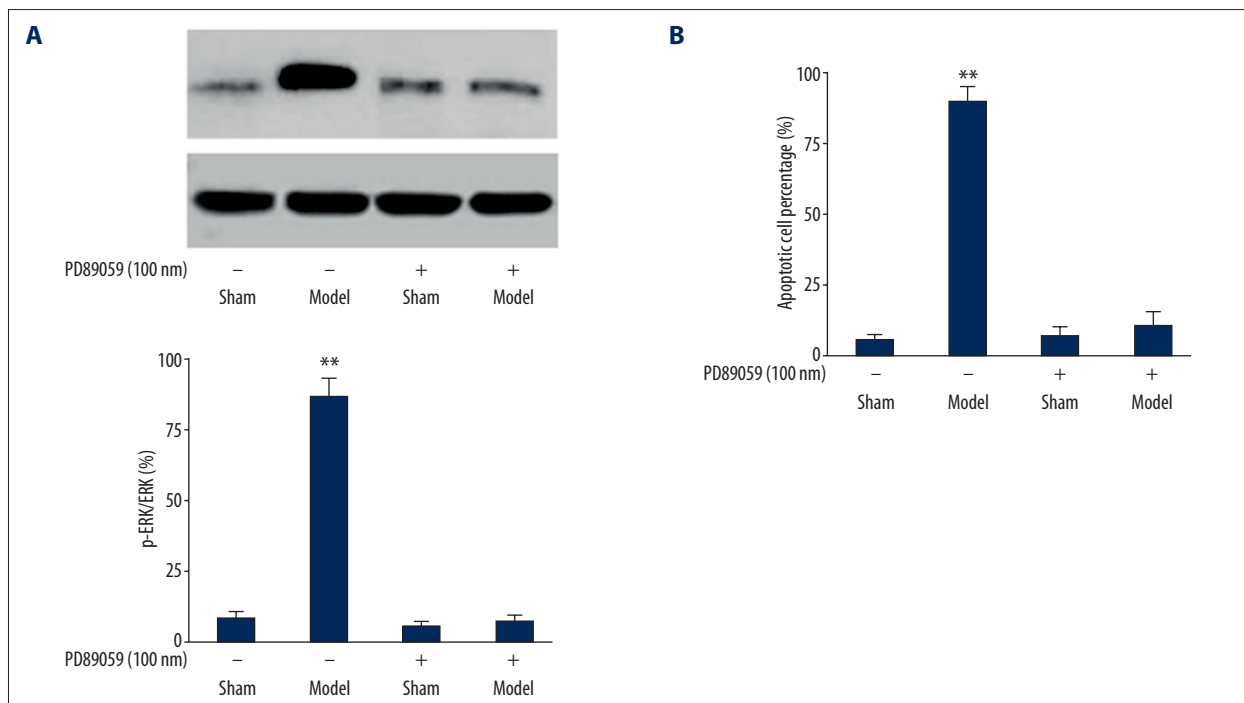


Figure 6. ERK pathway and cell apoptosis. (A) PD89059 blocked the phosphorylation of ERK; (B) PD89059 treatment decreased apoptotic cell ratio *in vitro*. One-way analysis of variance (ANOVA) was used to compare means across groups. The ** $P < 0.01$ represents the comparison of apoptosis cell percentage or p-ERK/ERK value in the model group vs. the sham group.

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