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Research Article

Downregulation of miR-568 in Atrial Fibrillation Leads to Increased Expression of NAPMT and TRMP7

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Abstract

Aim: Atrial fibrillation (AF) is known as the most common permanent cardiac arrhythmia worldwide with its incidence and prevalence gradually increase with age and cause significant morbidity and mortality. However, the epigenetic alterations underlying the development of this disease remains less understood. MicroRNAs (miRNAs), as one of the epigenetic regulators, are small non-coding RNAs that can target multiple genes to modulate proteins in different signaling pathways. Current studies have demonstrated that miRNAs, which are pivotal regulators of gene expression, may be involved in the pathophysiology of AF. The current study aims to clarify the miRNA regulated cellular signaling in atrial fibrillation.
Material and Method: An AF model was generated by providing external electrical stimulation to the HL-1 mouse cardiomyocyte cell line for 24 hours in this study. To understand the molecular mechanisms of miRNAs underlying the AF model, miRNA microarray analysis was performed. The gene sets obtained from the microarray analysis and the bioinformatically obtained putative targets were intersected and pathway enrichment analysis was performed. qRT-PCR was performed for validation of the selected miRNAs and potential targets.
Results: miRNA expression profile changes between the control group without external stimulation and the samples at the end of 3-, 6-, 12- and 24-hour stimulation were compared with microarray analysis. In particular, our transcriptomic analysis showed 5 distinctively expressed miRNAs (DEmiRNAs) whose target genes are associated with cardiovascular development within the stimulated groups in HL-1 cells. Additionally, our bioinformatics analysis revealed that targets of these miRNAs are concentrated in biological processes associated with cardiovascular development: smooth muscle cell proliferation, muscle cell proliferation, cell morphogenesis involved in differentiation and regulation of cell differentiation. Specifically, qPCR-based analyses confirmed the inverse correlation of miR-568 and potential targets of this miRNA. While miR-568 expression decreased with prolonged stimulation, expression of its potential targets, NAMPT and TRPM7, increased during prolonged stimulation.
Conclusion: This study supported the potential regulative role of miRNAs and their targets in the development of AF.

Keywords: Atrial fibrillation, miR-568, NAMPT, TRPM7

INTRODUCTION

Atrial fibrillation (AF) is known as the most common arrhythmia in the clinic, occurring with uncoordinated atrial contractions as a result of irregularities in atrial activation (1,2). AF is linked to atrial remodeling of the left atrium (3), and it is unclear whether AF is the ultimate consequence of atrial remodeling caused by heart disease combined with harmful environmental factors or is the main cause of progressive remodeling. AF and its complications are accountable for significant morbidity and mortality in adults. Like other cardiac arrhythmias, physiological approaches have been tried to be developed to explain the etiology of AF, but over time it has been seen to have a complex pathogenesis and molecular approaches have begun to be developed in the last two decades. Although the electrophysiological and molecular changes occurring in cardiac tissue have been described by studies, the molecular mechanism needs to be elucidated in more detail in order to develop therapeutic strategies for AF (4).

From a pathophysiological standpoint, AF is primarily characterized by atrial electrical remodeling involving ion channel alterations (5) , as well as structural remodeling (fibrosis and apoptosis) that supports the recurrence and persistence of arrhythmias (6). Atrial arrhythmogenic

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remodeling, characterized by changes in atrial structure or function that facilitate atrial arrhythmias, is a fundamental aspect of AF. This remodeling can stem from pre-existing cardiac conditions, systemic influences or factors like aging or the presence of AF itself. Recent studies have underscored the significance of remodeling in AF, offering new insights into fundamental mechanisms, and novel biomarkers are being developed to monitor remodeling processes based on these findings. Notably, the importance of abnormalities in intracellular Ca2+ processing has been emphasized, both for inducing ectopic activity and activating Ca2+-related cell signaling that drives profibrillator remodeling. Furthermore, the involvement of microRNAs (miRNAs), a recently identified category of small noncoding RNA sequences that control gene expression, has become apparent in both electrical and structural remodeling.

Recent investigations have unveiled the significant role of miRNAs in the onset and progression of cardiovascular disease (7). miRNAs, a group of small non-coding RNAs with high conservation, oversee nearly all cellular processes by exerting post-transcriptional control. They achieve this by binding to complementary sequences within the 3'-UTR region of target mRNAs, subsequently instigating the degradation or inhibition of translation for these mRNAs (8,9).

Given that these small RNAs can modulate multiple target mRNAs and that several miRNAs can target candidate mRNAs, the dysregulation of miRNAs is understandably a hallmark of many pathological conditions. miRNAs are employed as disease biomarkers due to their tissue- and pathology-specific expression. The functional miRNAs have been offered as potential targets for diverse cardiac conditions and arrhythmogenesis (10-12). It is crucial to establish direct cause-and-effect relationships between miRNAs and their mRNA targets to comprehend the molecular mechanisms driving diseases and to formulate precise therapeutic interventions. Increasing evidence indicates that miRNAs regulate processes such as modulation of Ca2+ handling, controling cardiac excitability and cardiac conduction (13,14).

The stability and detectability of miRNAs in biofluids, including peripheral blood, position them as promising diagnostic biomarkers for various cardiovascular diseases. However, further investigations are required to assess circulating miRNAs as dependable biomarkers for AF. Studies centered on miRNAs hold considerable value in comprehending the mechanisms driving AF, evaluating miRNAs' potential roles as biomarkers, and advancing miRNA-based therapies.

The HL-1 cell line, derived from cardiac muscle, exhibits a gene expression pattern resembling that of typical mice atrial cardiomyocytes. In addition to preserving their capacity to divide without reverting to an embryonic state, HL-1 cells preserve cardiac morphology properties, electrophysiological characteristics and biochemical attributes (15). In this study, we appraised the transcriptomic profile of miRNAs and relation with potential gene targets in AF using the HL-1 cells.

MATERIAL AND METHOD

Cell Culture and Generation of AF Model

The HL-1 cell line was purchased from Sigma-Aldrich (Cat. No: SCC065, St. Louis, Missouri, USA), and cells were cultured in Claycomb medium (Sigma, 51800C) supplemented with 10% fetal bovine serum (FBS) (Sigma, F7524, St. Louis, Missouri, USA), 1% penicillin/streptomycin (Sigma, P4333, St. Louis, Missouri, USA), 0.1 mM Norepinephrine (Sigma, A0937, St. Louis, Missouri, USA), and 2 mM L-Glutamine (Sigma, G7513, St. Louis, Missouri, USA). Flasks were coated with 3 mL of gelatin (Sigma, G9391, St. Louis, Missouri, USA)/fibronectin (Roche, 10838039001, Basel, Switzerland) for 1 hour in a humidified atmosphere at 37°C and 5% CO_2.

For the AF model, cells were seeded into 6-well plates, and using the carbon electrode IonOptix C-Dish™ integrated with the IonOptix C-Pace EP Cell Culture Stimulator 100 pacer system, a 5 Hz for 4 s (P1 wave) and 1 Hz for 1 s at 1.5V/cm^3 current per well (P2 wave) was applied to the cells. HL-1 cells, seeded at $1x10^6$ per well in 6-well plates, were stimulated for 3, 6, 12, and 24 hours. Electrical capture in the stimulated cells was confirmed using microscopy, and the data are provided in Figure 1.

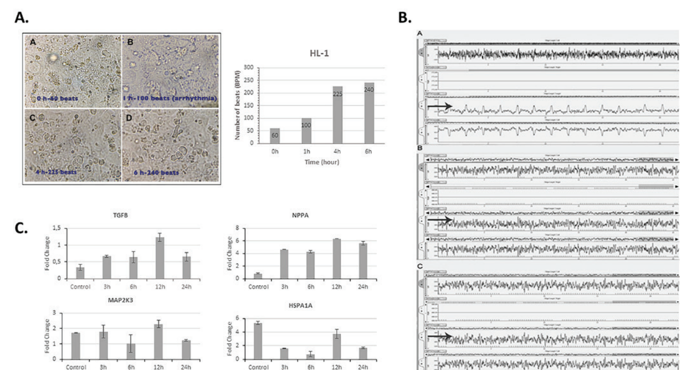

Figure 1. Supportive information of our AF model, **1A.** Microscope images of HL-1 cells as a result of electrical stimulation and change in the number of pulses made by HL-1 cells depending on the time of electrical stimulation, **1B.** Electrocardiogram images of cells stimulated in AF modeling. (a) represents control group, (b) represents 3rd hour and (c) represents 6th hour. **1C.** The onset of transcriptomic changes in response to stimulation were analysed in qRT-PCR ($p<0.05$)

RNA Isolation

Briefly, cells were homogenized using Qiazol reagent (Qiagen, 79306, Hilden, Germany), and chloroform (Sigma, C2432, St. Louis, Missouri, USA) was added and vigorously mixed. The samples were then incubated at room temperature for 2 minutes and centrifuged at 13,300 rpm for 15 minutes at 4˚C. After collecting the supernatant aqueous phase into a fresh tube, isopropanol (Sigma, I9516, St. Louis, Missouri, USA) was added, and the mixture was centrifuged again at 13,300 rpm for 15 minutes at 4˚C. Subsequently, the samples were washed twice with 70% ethanol. The RNA integrity of the samples was then assessed.

Transcriptome Analysis

The analysis of miRNA expression was performed on total RNA extracted from cells in the unstimulated control group, with samples taken at four different stimulation times (3rd, 6th, 12th, and 24th hours). Microarray procedures were carried out according to the Affymetrix Whole Transcript (WT) Expression Arrays instruction manual. Briefly, 250 ng of total RNA extracted from cells was labeled with the Whole Transcriptome Reagent Kit (ThermoScientific, P/N 902280, Massachusetts, USA). The labeled samples were then purified and hybridized with the Mouse Gene 2.0 array (ThermoScientific, Massachusetts, USA). Arrays were scanned on the Affymetrix Scanner 7G, and image analysis was performed using Expression Console™ Software v1.4 (ThermoScientific, Massachusetts, USA), followed by data normalization and analysis using BRB-ArrayTools.

Quantitation of miRNA and mRNAs

Total RNA was isolated using Qiazol reagent (Qiagen, 79306, Hilden, Germany). For the detection of miRNA expression, 1 µg of RNA was converted to cDNA with the Mir-X miRNA First-Strand Synthesis Kit (Takara, 638313, Kusatsu, Shiga, Japan). The TB Green Advantage® qPCR Premix was then employed along with specific primer sets for mmu-miR-568. RNU6 was used as an internal reference for analysis.

To detect mRNA expression, cDNA was generated from 1 µg total RNA using the First Strand cDNA Synthesis Kit (Roche, 11483188001, Basel, Switzerland). GAPDH was employed for coding gene normalization. qRT-PCR was performed using the LightCycler® 480 Real-Time System (Roche, Basel, Switzerland). Primers for miR-568 and coding genes are provided in Table 1.

Table 1. Sequences of primers for GAPDH, PIK3CA, NAMPT, TRPM7, RBM20, miR-568 and RNU6

Gene ID	Sequences (5'to 3')	Amplicon (bp)
PIK3CA	F: ACGACCATCTTCGGGTGAAC R: GTTTGATGGTGACGAGTGTGG	131 bp
GAPDH	F: TGTGTCCGTCGTGGATCTGA R: TTGCTGTTGAAGTCGCAGGAG	150 bp
NAMPT	F: CCACCGACTCGTACAAGGTT R: TGTTTCCTCGTATTTCACCTTCCT	131 bp
TRPM7	F: TCTGTGGTCGTTTGGTCAAGCA R: TGTTGGGCTCTGCTCCGTG	140 bp
RBM20	F: AGAGATGCCTGCGTTATGCC R: GGAGCTTGGCAGCATTTTGG	139 bp
mmu-miR-568	F: GTGGGGGGATGTATAAATGTAT R: CTCGCTTCGGCAGCACA	-
RNU6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT	-

Bioinformatic Analysis

Screening DEmiRNAs

Differentially expressed miRNAs (DEmiRNAs) were screened using the Excel-based software package BRB-ArrayTools. This tool aids in identifying DEmiRNAs between groups of samples and controls. In the present study, DEmiRNAs between four different stimulation times and the unstimulated control group were selected based on statistical significance ($p<0.05$ and $|logFC|>0.5$).

The clustering of the DE miRNAs and visualization of the clustering via heatmap was also performed by BRB-ArrayTools.

Target prediction analysis

To identify predictive targets for miR-568, MiRWalk was utilized. Additionally, TargetScan was employed to identify the 3'UTR binding sites of the miR-568-targeted genes. The list of AF-related genes was obtained from NCBI-Gene (https://www.ncbi.nlm.nih.gov/gene).

Statistical Analysis

Statistical significance was determined using Student's t-test (p value of <0.05) and the data are presented as the mean+SD.

RESULTS

AF Model Confirmation

To assess the AF pattern in the HL-1 cell line, electrical stimulations were observed for 24 hours under specific current and conditions. As a control, the number of pulses was measured before subjecting HL-1 cells to electrical stimulation, revealing a consistent rate of 60 pulses per minute (bpm). No change in the beat frequency was observed during the initial 30 minutes. However, starting from the 1st hour, the cells exhibited an increase in beats to 100 bpm, and irregularities in beat rhythm became apparent. Subsequently, the number of beats reached 105 bpm after 90 minutes, approximately 225 bpm at the end of the 4th hour, and around 240 bpm at the end of the 6th hour, as documented in microscope records (Figure 1A). This value aligns with the occurrence of arrhythmic beats (approximately 300 bpm) observed in atrial fibrillation patients.

To evaluate the potential development of an AF model

following electrical stimulation applied to HL-1 cells, electrocardiogram images were recorded using IonOptix Myocyte Calcium Photometry and Contractility Systems (Figure 1B). According to the obtained records, regular waves were observed in the control group cells that were not stimulated, while irregularities were detected in the cell pulse waves in the 3rd and 6th hour samples of stimulation.

In addition to electrocardiogram analysis, qRT-PCR analysis was performed using *HSPA1A, TGFB, NPPA,* and *MAP2K3* genes, whose expression profiles are reported to change in atrial fibrillation in the literature. As illustrated in Figure 1C, significant changes were detected as early as 3 hours after the initiation of rapid stimulation. The onset of transcriptomic changes in response to stimulation suggests the establishment of the AF pattern in these cells.

Analysis of DEmiRNA in HL-1 Cells

Among the differentially expressed miRNAs in response to each stimulation time, (3h, 12h and 24 h) 5 miRNAs were found to be common, which were miR-568, miR-let7c-1, miR-145a, miR-490 and miR-505 (Figure 2A). Based on the heatmap analysis, miR-568, let-7c-1, and miR-145a exhibited increased level of expression during the initial 3 hours of stimulation. Subsequently, their expression levels gradually declined in a time-dependent manner, extending from 3 hours to 24 hours (Figure 2B). We also confirmed the decreasing expression pattern of miR-568 by qRT-PCR in control and 24 h stimulated cells (Figure 2C). It may also important to note that the mature sequence of miR-568 was found to be evolutionary conserved between human, mouse and rat (Figure 3).

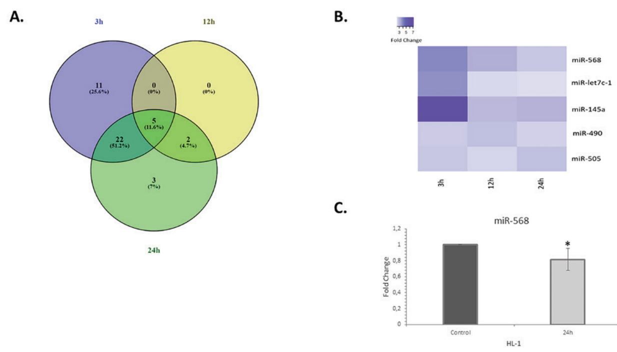

Figure 2. DEmiRNA screening, **2A.** According to intersection analysis of samples with different stimulation time, 5 common differential expressed miRNAs was shown, **2B.** The heatmap graph containing the varying expression values of the 5 common microRNAs. **2C.** Down-regulation of miR-568 was confirmed by RT-PCR (n=3, *p<0.01)

miR-568 Sequences

Human	gauauacacuauauu**AUGUAUAAAUGUAUACACAC**uuccuauauguauccacauauauauagu
Mouse	acacuauauu**AUGUAUAAAUGUAUACACAC**uucuauauauguccacauauaugcggugugugu
Rat	guauacacuauauu**AUGUAUAAAUGUAUACACAC**uucuauaugugcccacauauauacagugu

Figure 3. miR-568 mature sequence conserved between human, mouse and rat. Multiple sequence alignment of the section of quasi-alignment for the species showing perfect alignment and sequences which is labeled blue one is mature form of miRNA

To elucidate the role of miR-568 in HL-1, we gathered its target genes using various databases, such as miranda, miRDB, miRWalk, and Targetscan. A total of 428 targets were identifed and their enrichment in biological processes was assessed using Gene Ontology analysis. Among the GO analysis results, our emphasis was on the significant processes associated with cardiovascular development, such as the regulation of smooth muscle cell proliferation (FDR=0.022013, p value=0.0000040424) and muscle cell proliferation (FDR=0.034534, p value=0.0000082563), cell morphogenesis involved in differentiation (FDR=0.0010253, p value=3.1379e-8), and regulation of cell differentiation (FDR=0.0013541, p value=7.4599e-8).

Pathway enrichment analysis was performed to delve into the molecular functions of the targets associated with miR-568 by using KEGG. The enrichment analysis of identified gene targets for miR-568 revealed involvement in various KEGG pathways, notably the cAMP signaling (FDR=0.030316, p value=0.000095936), mTOR signaling (FDR=0.023211, p value=0.000073219), FoxO signaling (FDR=0.00013671, p value=4.2064e-7) and Rap1 signaling (FDR=0.037255, p value=0.00011903).

miR-568 Enhances the Expression of AF-Related Genes in HL-1 Cells

To demonstrate the potential role of miR-568 in AF, we intersected the targets of miR-568 with the AF-related gene list and found 4 genes (*NAMPT, TRPM7, RBM20* and *PIK3CA*) in common (Figure 4A). qRT-PCR results showed that the expression of AF-related genes, including *NAMPT* and *TRPM7* were significantly increased in 4 different stimulation times in a time dependent manner. Meanwhile, expression of *RBM20* and *PIK3CA* were markedly decreased in stimulation groups (Figure 4B). The converse expression patterns of miR-568 and its targets NAMPT and TRPM7 at the same time points supported the potential role of them in AF and led us to identify the interaction between these miRNA and target genes.

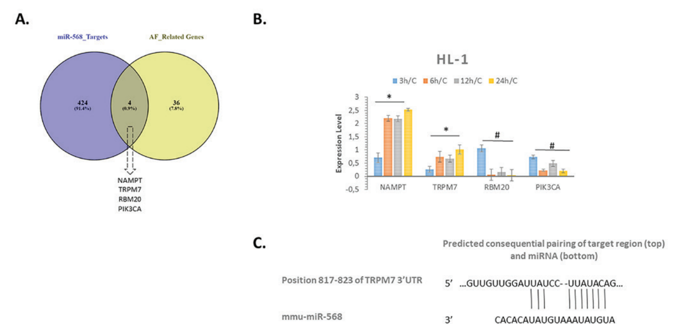

Figure 4. TRPM7 is a target gene of miR-568, **4A.** Identification of NAMPT, TRPM7, RBM20 and PIK3CA as a common target of miR-568, **4B.** qRT-PCR was performed to determine the expression levels of predicted target genes of miR-568 in HL-1 cells, **4C.** Predicted mmu-miR-568 (MI0005517) seed match to the sequence in the 3'UTR of TRPM7 mRNA. The seed sequences of miR-568 are AAUAUGU

miR-568 Interacts with 3-UTR of TRPM7 Gene

Analysis of the TargetScan showed that *TRPM7* was a potential target gene of miR-568. The matching positions for miR-568 within 3'-UTR of the targeted *TRPM7* mRNA are shown in Figure 4C.

DISCUSSION

AF is the most common clinical arrhythmia and by 2060 it is anticipated to attain a prevalence of 18 million in Europe (16). Patients with atrial fibrillation experience higher mortality rates and current therapeutic options do not fully address the clinical need for AF. Exploring the cellular mechanisms behind AF and devising innovative strategies to enhance the survival of individuals with AF are promising avenues for future research.

Over the past decade, substantial strides have been made in understanding AF and its underlying mechanisms, leading to the identification of numerous genetic (17-19) and epigenetic factors (20,21). In this context, miRNAs have recently been included among the molecular factors linked to the progression of AF, particularly in electrical and structural remodeling, that represent the two primary elements of atrial remodeling caused by fibrosis (22,23). Associations between cardiovascular diseases and miRNAs have been accelerated in recent years. A number of miRNAs have been described to be differently expressed and to modulate signaling pathways during cardiovascular diseases. It has been showed that miR-208 could act as a therapeutic molecule in cardiac fibrosis, heart failure and myocardial hypertrophy (24). Collectively, miR-595 and miR-155 exhibited a positive correlation with the left ventricular mass (LVM) index, while miR-182, miR-200a, and miR-568 demonstrated a negative correlation with the LVM index (25).

miR-568 was one of the differentially expressed miRNAs in response to stimulation in our study and its downregulation through increased stimulation times was confirmed by qRT-PCR. Meanwhile its putative targets, TRPM7 and NAMPT showed increased expression patterns which is expected since the miRNA-target expression patterns are inversely correlated. These findings may support the potential role miR-568 together with its targets TRPM7 and NAMPT in the development of AF. Among TRP channels, TRPM7 is particularly abundant in heart tissue (26,27) and is highly expressed myocardium during embryonic stage (28). Intercellular ICa transitions are greatly altered in atrial myocytes during AF. The reshaped regulation of calcium within cells is a significant factor in the emergence of impaired contractile function and alterations in atrial electrophysiology, specifically the remodeling of both contractile and electrical aspects, which are distinctive features of AF. TRPM7 current has recently been observed to undergo upregulation in atrial fibrillation. It is suggested that TRPM7 may serve as a calcium entry pathway, triggering TGF-β1 signaling, thereby contributing to the development of atrial pathogenesis (29). NAMPT exhibits high expression in cardiomyocytes, and recent studies have demonstrated its impact on various cardiac conditions such as dilated cardiomyopathy, ischemia/reperfusion injury, and heart failure (30-32). However, there is a lack of reports on the correlation between AF and Nampt. Given the association of the Nampt/NAD axis with obesity and aging, crucial risk factors for AF, it is postulated that the Nampt/NAD axis plays a significant role in the pathogenesis of obesity-related AF (33). The RNA-binding motif protein 20 (RBM20) gene, serves as a splicing factor (34) and is notably expressed in striated muscles, particularly the heart, and has been shown to significantly impact the splicing patterns of key genes essential for cardiac function (35,36). The manifestation of RBM20 cardiomyopathy is marked by a deeply penetrating and aggressive nature, closely linked to sudden cardiac death arrhythmias and heart failure (37,38). More than 30 genes have now been shown to be targets of RBM20. This encompasses additional genes vital for sarcomeric function, along with numerous genes essential for the efficient utilization of calcium in cardiomyocytes (39).

The complexity of the arrhythmia known as AF encompasses its genetic bases and inheritability. Significant advancements have been made in various areas, including risk assessment for AF, the discovery of new therapeutic targets, and the development of prediction models based on the genome. It is evident that gaining a deeper insight into the molecular mechanism of AF will not only enhance prediction models for the condition but will also pave the way for more effective personalized treatment approaches.

In this study, we generated an AF model in HL-1 cells through a directed electrical stimulation protocol that predominantly exhibits atrial-like electrophysiologic properties. This work involves characterizing and interrogating the genome-wide profiling of miRNA expression in atrial fibrillation. miR-568 expression was downregulated in HL-1 cells. Using bioinformatics approaches, we demonstrated a potential relation of miR-568 with cardiovascular development and related signaling pathways. miRNAs, including down-regulated miR-568, miR-let-7c-1, and miR-145a, as well as certain genes, including *NAMPT, TRPM7, RBM20,* and *PIK3CA,* might be related to a greater risk of AF.

CONCLUSION

It could be concluded that miR-568 may involve in the AF development or the impact of AF through regulating its putative targets *NAMPT* and *TRPM7*. Given that the mature sequence of miR-568 is conserved in humans, it would be valuable to confirm these data in human samples with further studies.

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