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ORIGINAL ARTICLE
Arrhythmia/Electrophysiology

Atrial Fibrillation-Mediated Upregulation of miR-30d Regulates Myocardial Electrical Remodeling of the G-Protein-Gated K+ Channel, $I_{K.ACh}$

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Background: Atrial fibrillation (AF) begets AF in part due to atrial remodeling, the molecular mechanisms of which have not been completely elucidated. This study was conducted to identify microRNA(s) responsible for electrical remodeling in AF.

Methods and Results: The expression profiles of 1205 microRNAs, in cardiomyocytes from patients with persistent AF and from age-, gender-, and cardiac function-matched control patients with normal sinus rhythm, were examined by use of a microRNA microarray platform. Thirty-nine microRNAs differentially expressed in AF patients' atria were identified, including miR-30d, as a candidate responsible for ion channel remodeling by in silico analysis. MiR-30d was significantly upregulated in cardiomyocytes from AF patients, whereas the mRNA and protein levels of *CACNA1C*/Cav1.2 and *KCNJ3*/Kir3.1, postulated targets of miR-30d, were markedly reduced. *KCNJ3*/Kir3.1 expression was downregulated by transfection of the miR-30 precursor, concomitant with a reduction of the acetylcholine-sensitive inward-rectifier K^+ current ($I_{K.ACh}$). *KCNJ3*/Kir3.1 (but not *CACNA1C*/Cav1.2) expression was enhanced by the knockdown of miR-30d. The Ca^{2+} ionophore, A23187, induced a dose-dependent upregulation of miR-30d, followed by the suppression of *KCNJ3* mRNA expression. Blockade of protein kinase C signaling blunted the $[Ca^{2+}]_i$-dependent downregulation of Kir3.1 via miR-30d.

Conclusions: The downward remodeling of $I_{K.ACh}$ is attributed, at least in part, to deranged Ca^{2+} handling, leading to the upregulation of miR-30d in human AF, revealing a novel post-transcriptional regulation of $I_{K.ACh}$. (*Circ J* 2016; **80:** 1346–1355)

Key Words: Atrial fibrillation; $I_{K.Ach}$; miRNA microarray; miR-30d; Remodeling

Atrial fibrillation (AF), the most common cardiac rhythm disorder, is a major cause of cardiovascular morbidity and mortality.[1] AF is characterized by the rapid and irregular activation of the atrium[2] with diverse abnormalities, including electrical, structural, metabolic, neurohormonal, or molecular alterations.[3] Although the pathophysiology of AF is complex, it has traditionally been treated with antiarrhythmic drugs that control the rhythm by altering cardiac electrical properties, principally by modulating ion channel function.[3,4] However, treatment for AF with antiarrhythmic drugs have usually failed to control the rhythm or the pharmacological effect is limited, because the electrical characteristics of atrial cardiomyocytes are eventually altered or remodeled during the course of AF.[2,3] Atrial electrical remodeling is characterized by a marked shortening of the action potential duration (APD) and refractoriness.[3,5] In cardiomyocytes from patients with AF (hereafter referred to as AF cardiomyocytes), the amplitude of the L-type Ca^{2+} currents ($I_{Ca.L}$) and the transcription of the gene (*CACNA1C*) encoding Cav1.2 decreased, which might engage a homeostatic defense mechanism against chronic Ca^{2+} overload.[6] In contrast, the inward-rectifier K^+ current (I_{K1}), along with expression of the principal underlying subunit *KCNJ2* mRNA and its encoded Kir2.1 protein, is upregulated in AF.[7] Because I_{K1} is one of the key K^+ currents responsible for setting the resting potential and APD in atrial cardiomyocytes, the augmentation of I_{K1} is an important factor favoring AF

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Table. Characteristics of Patients With NSR and AF

	NSR (n=19)	AF (n=14)	P value
Age, years	71.1±1.1	69.5±1.2	0.307
Male sex, n (%)	19 (100)	14 (100)	NS
Heart rate, beats/min	68.4±2.2	70.1±4.2	0.756
Systolic blood pressure, mmHg	123.6±4.5	116.9±4.2	0.123
Diastolic blood pressure, mmHg	65.0±2.9	68.0±3.5	0.574
BNP (pg/ml)	285.6±80.9**	337.1±104.7†	0.180
Echocardiographic parameters			
LAD, mm	39.7±1.8	47.5±2.8	0.080
LVEDD, mm	53.0±1.8**	54.4±3.8††	0.752
LVPW, mm	10.6±0.7**	11.0±1.1††	0.882
LVEF, %	65.8±1.9	61.3±3.3	0.132
History of medicine			
ARB, n (%)	8 (42)	9 (64)	0.296
Ca^{2+} blocker, n (%)	10 (53)	7 (50)	1.000
β-blocker, n (%)	1 (5)	3 (21)	0.288
Diuretic, n (%)	5 (26)	11 (79)	0.011*
Digitalis, n (%)	1 (5)	2 (14)	0.561
Statin, n (%)	7 (37)	1 (7)	0.098
Anti-arrhythmic agent, n (%)	0 (0)	3 (21)	0.067

*P<0.05 compared with the NSR group. All values are expressed as mean±SE. The statistical difference was determined by using the Student's t-test and by Fisher's exact test for categorical variables. Data are from **16 NSR patients, †11 AF patients, and ††12 AF patients, respectively. AF, atrial fibrillation; ARB, angiotensin II receptor blocker; BNP, B-type natriuretic peptide; LAD, left atrial dimension; LVEDD, left ventricular end diastolic dimension; LVEF, left ventricular ejection fraction; LVPW, left ventricular posterior wall; NS, not sig nificant; NSR, normal sinus rhythm; SE; standard error.

maintenance.[7] Another important inward-rectifier current, the acetylcholine-sensitive K^+ channel current ($I_{K.ACh}$), which consists of the Kir3.1 (*KCNJ3*) and Kir3.4 (*KCNJ5*) gene products, also appears to be responsible for regulating the APD and refractory period. Dobrev et al showed that chronic human AF induced transcriptional downregulation of $I_{K.ACh}$ to counteract the shortening of the atrial effective refractory period due to electrical remodeling.[8] Although the abnormal function or expression of these ion channels as a molecular mechanism in the basis of arrhythmogenesis has attracted a great amount of attention from researchers, the molecular mechanisms that regulate ion channel genes in atrial cardiomyocytes during AF have not been completely elucidated.

A rapidly emerging area of study on the pathophysiology of AF is the role of microRNAs (miRNAs or miRs), which are endogenous small non-coding RNAs (~22 nucleotides), which exert a role in the negative regulation of gene expression at the post-transcriptional level by binding to the 3´-untranslated region (3´-UTR) of the target mRNA to promote its degradation or to block translation.[9] Changes in cardiac miRNA expression profiles in response to pathological conditions are believed to play a pathological role in various cardiac diseases, including AF.[10–12] Of special interest are the contributions of miRNAs to a wide range of cardiac electrical remodeling processes.[10] Recent studies have demonstrated that Cav1.2, which is a target of miR-328 and miR-21, was markedly decreased in cardiomyocytes with chronic AF, accompanied by a decrease in $I_{Ca.L}$, suggesting that miR-328 and miR-21 contribute to AF-induced electrical remodeling.[13,14] Post-transcriptional regulation of Kir2.1 (*KCNJ2*) by miR-1 and miR-26 was also demonstrated in AF cardiomyocytes.[7,15] However, little is known about the molecular mechanisms regulating other ion channels in AF cardiomyocytes.

The aims of this study were to characterize the global changes in miRNA expression in human atrial appendages and to identify the target ion channel(s) responsible for electrical remodeling in persistent AF. In this study, we carefully selected patients that strictly represent samples to elucidate AF-specific changes in the miRNAs of the human atrium, and we propose the first evidence of miR-30d as a candidate of post-transcriptional mediator for $I_{K.ACh}$ remodeling in cardiomyocytes caused by persistent AF.

Methods

The study was approved by the ethics committees of Oita University (No. P-14-03) and conforms to the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent. Clinical data of the patients are included in Table and Table S1.

Human Tissue Samples

Human right atrial appendage was obtained from 14 patients with persistent AF (AF group) and 19 patients with normal sinus rhythm (NSR group) undergoing open-heart surgery at Oita University Hospital. Tissue samples were immediately frozen in liquid nitrogen, and then stored in a deep freezer at –80°C.

Cell Isolation and Culture

All animal experiments conformed to the Guidelines for the Care and Use of Animals at Oita University, and the NIH guidelines were approved by the institutional committee. Single neonatal rat cardiomyocytes were enzymatically isolated and cultured as previously described.[16]

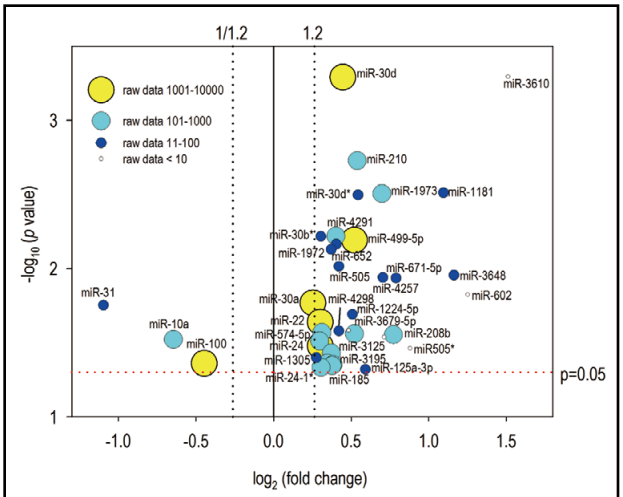

Figure 1. Volcano plot analysis of 39 microRNAs (miRNAs) based on microarray data representing the global miRNA changes in atrial fibrillation (AF) vs. normal sinus rhythm (NSR) as seen in **Figure S1A** and **Table S3**. Each circle represents one miRNA, as indicated. The log-fold change of miRNAs in AF normalized to NSR reference miRNA is represented on the X-axis. The Y-axis shows the –log10 values of the P value. P values (0.05) and fold changes (1.2, 1/1.2) are indicated by dotted lines. The size of the circles represents the expression intensity of raw data in microarray analysis.

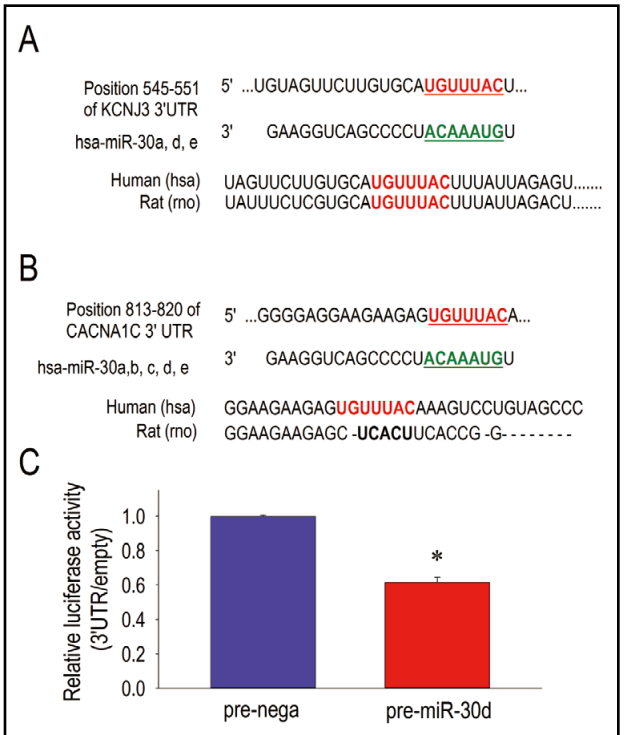

Figure 2. *KCNJ3* mRNA as a direct target of microRNA (miR)-30d. (**A**,**B**) Postulated target sites for miR-30d in the 3′-untranslated region (3′-UTR) of *KCNJ3* (**A**) and *CACNA1C* (**B**) in human (hsa) and rat (rno), as analyzed by TargetScan 6.2 software. (**C**) Interaction of miR-30d with the 3′-UTR of *KCNJ3*. After transfection with 3 nmol/L pre-miR-30d for 48 h, the reporter plasmid with *KCNJ3* 3′-UTR was significantly reduced by 37% in comparison with the pre-negative control, a reporter plasmid without *KCNJ3* 3′-UTR. Luciferase activities were measured 24 h after transfection of a plasmid expressing *Renilla* luciferase co-transfected with pre-miR30d or pre-negative control into HEK293 cells. *P<0.01 vs. negative control-transfected cardiomyocytes.

Total RNA Isolation

Total RNA was extracted from the collected atrial tissues with TRIzol reagents (Invitrogen) according to manufacturer's instructions.

Immunoblot Analysis

To measure Kir3.1 and Cav1.2 protein expression, an immunoblot analysis in human tissue or cardiomyocytes with miR-30d mimic was performed by using an anti-Kir3.1 or anti-Cav1.2 antibody (1:1,000; Alomone Labs, Jerusalem, Israel).

Fura-2 Ca^{2+} Measurement

Intracellular Ca^{2+} was monitored with the fluorescent Ca^{2+} indicator, fura-2 AM. Cultures were loaded with 0.3 μmol/L A23187 or vehicle for 24 h, following a load of 3 μmol/L fura-2 AM for 30 min at 37°C, then rinsed (twice) with extracellular solution, and allowed to de-esterify for 30 min before use. Ratiometric Ca^{2+} measurements were obtained by using a Nikon Eclipse TE2000 inverted microscope that was equipped with a filter-switching lambda DG-4 high-speed optical filter changer, a Hamamatsu OCRCA-ET digital CCD camera, and a Nikon S-Fluor ×40 oil immersion objective lens with a numerical aperture of 1.3. Dual images (340- to 380-nm excitation, 510-nm emission) were collected using HCImage software (Hamamatsu Photonics).

Statistical Analysis

Group data are expressed as mean±SE. Two-groups-only comparisons were performed by using the unpaired Student's t-test. Multiple-group comparisons were analyzed with one-way ANOVA, followed by Bonferroni's post hoc tests. Pearson's correlation test was used for the determination of correlations. P values of less than 0.05 were considered statistically significant, unless otherwise indicated.

Additional Information

Refer to the Methods section in the online-only Data Supplement for a detailed description of miRNA microarray, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for miRNA, real-time polymerase chain reaction (PCR) (**Table S2**), immunoblot analysis, electrophysiology and luciferase reporter assay.

Results

Patient Characteristics

Samples were only taken from male (60–79 years old) patients, and those with chronic heart failure, diabetes, inflammatory diseases, endocrine diseases, metabolic diseases, kidney diseases requiring hemodialysis, history of steroid treatment, and paroxysmal AF were excluded. The AF group included patients with a documented record of sustained AF for 6 months or longer. The NSR group included patients with no documented history of AF. The clinical backgrounds of the patients are shown in **Table** and **Table S1**. The 2 study groups had statistically identical clinical backgrounds except for the prescription ratio of diuretics.

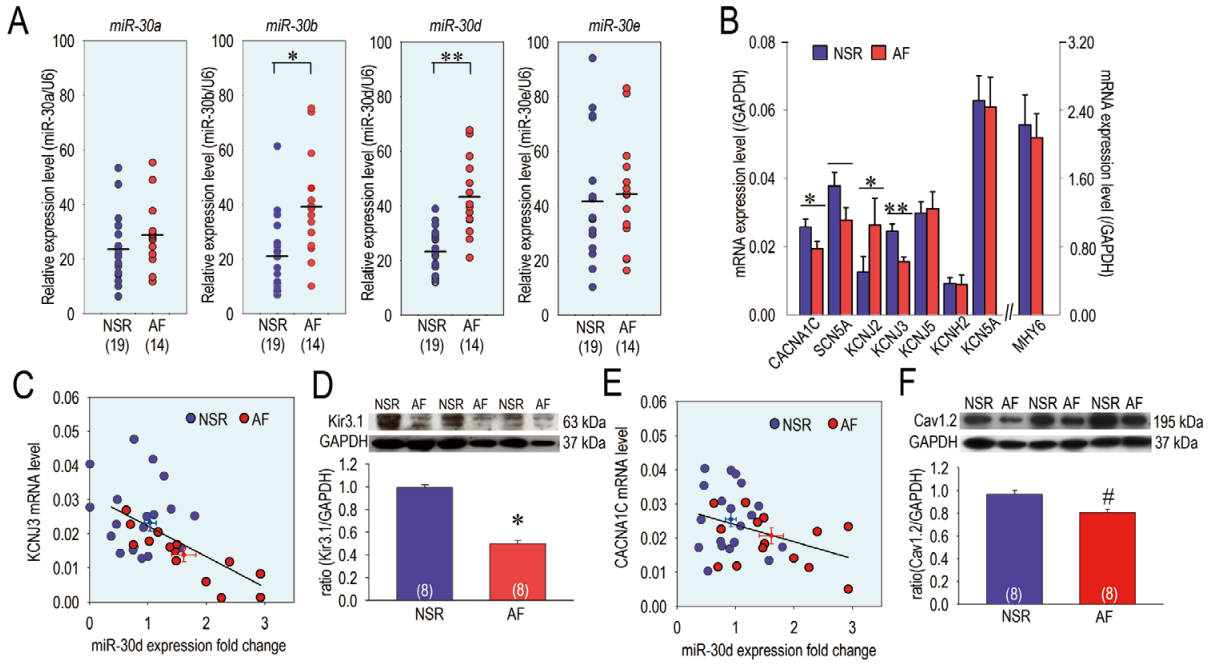

Figure 3. Expression of miR-30 family members and ion channels in human atrial cardiomyocytes. (**A**) Quantitative RT-PCR performed on microRNAs (miRNAs) in atrial cardiomyocytes from all 33 patients (n=19 for normal sinus rhythm (NSR) and n=14 for atrial fibrillation (AF)). Expression levels of miR-30 family members (miR-30a, miR-30b, miR-30d, and miR-30e) relative to U6 in atrial cardiomyocytes. Black lines represent the mean value for each group. (**B**) Changes in mRNA levels for *CACNA1C*, *SCN5A*, *KCNJ2*, *KCNJ3*, *KCNJ5*, *KCNH2*, *KCNA5*, and *MHY6* normalized to GAPDH in atrial cardiomyocytes (n=19 for NSR, and n=14 for AF). Data are expressed as the mean±SE. **P<0.01, *P<0.05, and †P=0.08 vs. the indicated groups. (**C**,**E**) Correlation between *KCNJ3* and miR-30d (**C**) and between *CACNA1C* and miR-30d (**E**) in terms of expression in NSR (purple circles) and AF (red circles) patients, as assessed by Pearson's correlation coefficient test. A statistically significant negative correlation (Pearson correlation coefficient r=–0.81, P=0.01) was observed between *KCNJ3* mRNA and miR-30d, but not between *CACNA1C* mRNA and miR-30d (r=–0.349, P=0.222) (**E**). Blue crosses and red crosses represent the mean±SE for the NSR and AF groups, respectively. (**D**,**F**) Representative protein expression and their group data for Kir3.1 (**D**) and Cav1.2 (**F**) in NSR and AF cardiomyocytes (n=8). Data are expressed as the mean±SE. *P<0.05 or #P=0.09 vs. NSR groups.

Identification of miRNAs Distinctly Expressed in Cardiomyocytes of AF Patients

We used a miRNA microarray platform, covering a total of 1,205 human miRNAs, to compare the expression profiles of miRNAs in atrial myocardia from 9 individuals with persistent AF with those from 11 NSR individuals (**Figure S1**, **Table S3**). Of the total 1,205 miRNAs studied, 355 were selected after filtering with an algorithm based on raw signal intensity (>20.0), finally yielding 39 miRNAs that were filtered by Welch's t-test (P<0.05), as shown in **Table S3**. The expression pattern of 39 miRNAs was examined by supervised hierarchical cluster analysis, indicating that the tree of AF patients was separated from that of NSR patients (**Figure S1B**). These 39 miRNAs were replotted according to their expression intensity, where miR-30d and miR-499-5p were particularly highly expressed in cardiomyocytes (**Figure 1**, **Table S3**). We investigated whether the whole distinct 39 miRNAs could interact with alignment of the 3´-UTR of 14 cardiac ion channels or its related genes (*SCN5A*, *CACNA1C*, *CACNA1G*, *CACNA1H*, *KCNJ2*, *KCNJ3*, *KCNJ5, KCNA5*, *KCND3*, *KCNH2*, *KCNIP2*, *KCNE1*, *KCNE2*, and *KCNQ1*) whose expression levels were assumed to be potentially changed during AF. In silico analysis by TargetScan 6.2 and other software found that miR-30d was identified as a candidate miRNA responsible for ion channel remodeling (**Table S4**). Interestingly, 2 ion channel genes, *CACNA1C* and *KCNJ3*, were predicted as direct targets of miR-30d (**Figure 2**). Notably, miR-30d was significantly upregulated (fold change of 1.36), with the least variability (P=0.0005 vs. NSR group) and the greatest intensity in the AF cardiomyocytes (**Figure 1**, **Table S3**).

MicroRNA-30d in Atrial Myocytes With AF

To validate the microarray data, the expression levels of miR-30 family members (miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e) in human cardiomyocytes were further analyzed by real-time PCR. Among the family members, miR-30b and miR-30d were significantly upregulated in the AF groups, although miR-30b expression levels did not appear to change upon microarray analysis. In contrast, the expression of miR-30a and miR-30e was not altered in the AF groups (**Figure 3A**). We also assessed changes in the expression of ion channel genes (*CACNA1C*, *SCN5A*, *KCNJ2*, *KCNJ3*, *KCNJ5*, *KCNH2*, and *KCNA5*) in AF cardiomyocytes from the same samples for comparison with those in the NSR groups. In AF cardiomyocytes, *CACNA1C*, *SCN5A*, and *KCNJ3* were significantly downregulated, whereas *KCNJ2* was significantly upregulated (**Figure 3B**). We also confirmed that *KCNJ3*, a putative target gene of miR-30d, was negatively correlated with miR-30d (r=–0.81, P=0.01) (**Figure 3C**). Consistent with the reduction of mRNA expression, the *KCNJ3*

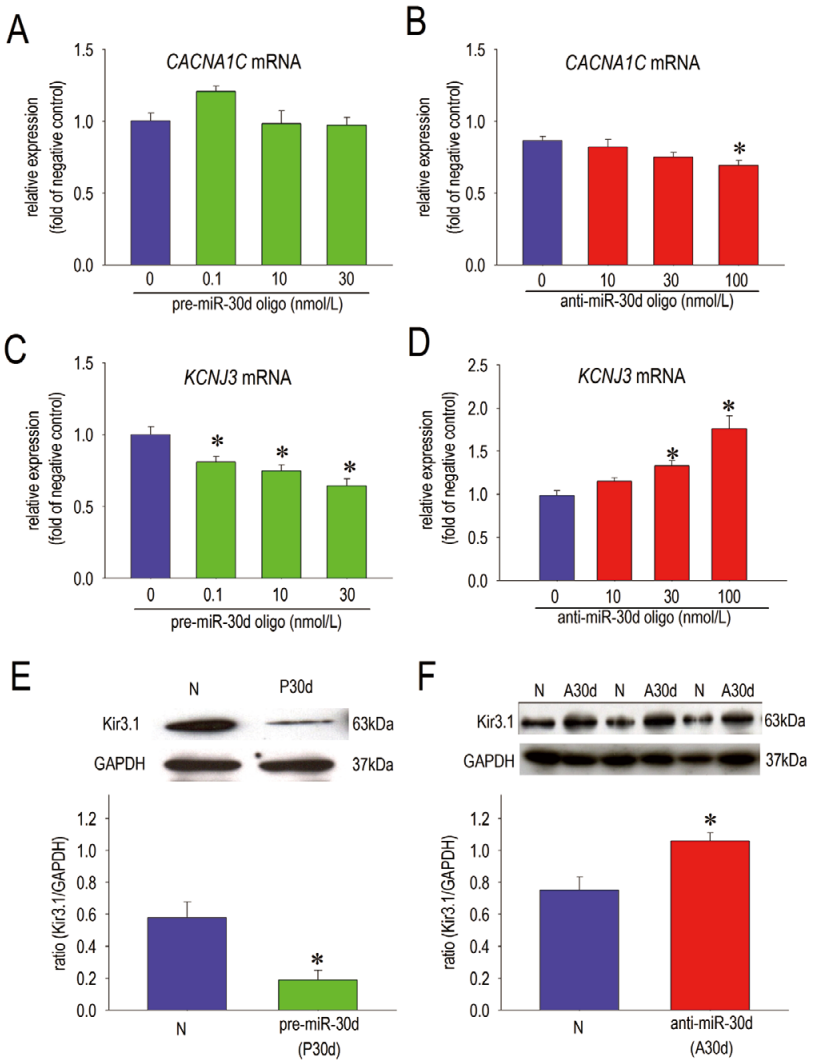

Figure 4. Modulation of the expression of *KCNJ3* (Kir3.1) and *CACNA1C* by pre-miR-30d or anti-miR-30d in neonatal rat cardiomyocytes. (**A**,**B**) Changes in *CACNA1C* mRNA by transfection with pre-miR-30d (**A**) or anti-miR-30d (**B**), as assessed by real-time PCR. (**C**,**D**) Changes in *KCNJ3* mRNA by transfection with pre-miR-30d (**C**) or anti-miR-30d (**D**). (**E**) Changes in Kir3.1 protein and its group data by pre-miR-30d (P30d), assessed at 48 h after transfection. (**F**) Changes in Kir3.1 protein and its group data by anti-miR-30d (A30d). Data are expressed as the mean±SE (n=8). *P<0.05 vs. absence of oligonucleotides or negative control (N).

protein (Kir3.1) was also significantly decreased in AF cardiomyocytes, suggesting a role of miR-30d in regulating *KCNJ3* (**Figure 3D**). *CACNA1C*, another potential target gene of miR-30d, was appreciably reduced in AF cardiomyocytes, with a relatively weak correlation to the expression of miR-30d (**Figures 3E**,**F**).

Validation of *KCNJ3* as a Target for MiR-30d

To determine the role of miR-30d in regulating *CACNA1C*/Cav1.2 and *KCNJ3*/Kir3.1 in cardiomyocytes, we next performed a series of functional assays using isolated neonatal rat cardiomyocytes. We examined the possible action of miR-30d on *CACNA1C*/Cav1.2 and *KCNJ3*/Kir3.1 mRNAs by transfecting the rat cardiomyocytes with precursor miR-30d (pre-miR-30d) and/or antisense inhibitor of miR-30d (anti-miR-30d) oligonucleotides. Contrary to our expectation, pre-miR-30d oligonucleotides failed to reduce the expression of *CACNA1C*, and anti-miR-30d oligonucleotides failed to increase its expression (**Figures 4A**,**B**). These results suggest a possibility that the upregulation of miR-30d in AF myocytes is unrelated to the reduction of Cav1.2. Unlike adult ventricular cardiomyocytes, neonatal rat ventricular cardiomyocytes are rich in *KCNJ3* (**Figure S2**). Transfection of pre-miR-30d into neonatal cardiomyocytes reduced the *KCNJ3* mRNA expression in a dose-dependent manner (**Figure 4C**). Consistently, Kir3.1 protein levels were also decreased by miR-30d overexpression (**Figure 4E**). Strikingly, upregulation of the *KCNJ3* mRNA and Kir3.1 protein was evident with the introduction of anti-miR-30d to knock down endogenous miR-30d (**Figures 4D**,**F**; **Figure S3**). To confirm the functional modulation of *KCNJ3* or Kir3.1 by miR-30d, $I_{K.ACh}$ or carbachol-sensitive current was recorded in neonatal cardiomyocytes with or without pre-miR-30d transfection (**Figure 5**). Cardiomyocytes transfected with pre-miR-30d demonstrated significantly smaller $I_{K.ACh}$ than those in control cardiomyocytes. Notably, the reduction of the inward component of $I_{K.ACh}$ by pre-miR-30d was comparable to the reduction of the outward component (**Figures 5B**,**C**). The luciferase reporter assay, performed to test whether miR-30d directly regulates the expression level of *KCNJ3*/Kir3.1 by binding to the putative target sequence in *KCNJ3*-3´-UTR, indicated that the expression of *KCNJ3* was highly sensitive to the miR-30d binding sequence (**Figure 2**).

Mechanism of MiR-30d Upregulation in AF Cardiomyocytes

To clarify the intracellular signal mechanism for miR-30d expression in cardiomyocytes from AF patients, we simulated the pathological condition of cardiomyocytes, such as Ca^{2+} overloading. Exposure of cardiomyocytes to the Ca^{2+} ionophore A23187 increased intracellular Ca^{2+} intensity approximately by 6-fold (**Figure 6A Inset**). This procedure induced the upregulation of miR-30d in a dose-dependent manner, which concomitantly decreased the *KCNJ3* mRNA levels (**Figures 6A,B,S4**). To further examine the action of intracellular Ca^{2+} overload, we explored the roles of calcineurin/nuclear factor of activated T-cells (NFAT) and/or protein kinase C (PKC) on miR-30d expression. The calcineurin inhibitors, cyclosporin A and FK506, did not affect the upregulation of miR-30d by A23187 (**Figure 6C**). Under the same experimental condition, a reduction of *KCNJ3* by A23187 was unaffected by FK506 (**Figure 6D**), although cyclosporin A blocked the effect of A23187 (**Figure 6D**) or reduced the expression of *KCNJ3* by itself at the high concentration of 100 μmol/L (**Figure S5**). In contrast, the upregulation of miR-30d by A23187 was abolished in the presence of the PKC inhibitors, chelerythrine and Gö6976 (**Figure 6E**), indicating that the upregulation of miR-30d by cellular Ca^{2+} overload was dependent on a classical $[Ca^{2+}]_i$-dependent PKC pathway. More strikingly, phorbol 12-myristate 13-acetate, an activator of PKC, significantly increased the expression of miR-30d (**Figure 6E**), which was thereby consistent with the reduction of *KCNJ3* in the same sample (**Figure 6F**).

Discussion

In the present study, we demonstrated a novel role for miR-30d in remodeling the cardiac ion channel in human persistent AF. MiR-30d was upregulated, whereas *KCNJ3*, a target gene of miR-30d, was downregulated in human AF cardiomyocytes. Overexpression of miR-30d suppressed the expression of *KCNJ3* mRNA and Kir3.1 protein, and consequently $I_{K.ACh}$ in cardiomyocytes. Conversely, knockdown of miR-30d upregulated the *KCNJ3* mRNA and Kir3.1 protein levels. Cellular Ca^{2+} overload increased the expression of miR-30d and thereby decreased the expression of *KCNJ3*, which was abolished by the inhibition of PKC activity. Furthermore, activation of PKC increased the miR-30d level and decreased *KCNJ3*. Our data provided the first evidence that the downward remodeling of $I_{K.ACh}$ in Ca^{2+}-overloaded cardiomyocytes is attributed, at least in part, to deranged Ca^{2+} handling, leading to the upregulation of miR-30d in human AF (**Figure 7**).

miRNAs and AF

The expression and/or function of many ion channels are altered by atrial electrical remodeling during AF.[17,18] Recent studies have demonstrated various mechanisms for the deregulation of gene expression in cardiomyocytes from AF patients, including transcriptional modulation and post-transcriptional modulation of ion channels. Unlike transcription factors, miRNAs primarily silence gene expression by binding to the 3´-UTR of various genes to modulate the proteome of cells. Emerging evidence suggests that miRNAs regulate the genes encoding cardiac ion channels, transporters, Ca^{2+}-handling proteins, and other relevant proteins associated with AF substrates and triggers.[19–22] Although the molecular mechanisms underlying post-transcriptional ion channel remodeling in AF cardiomyocytes remain unresolved, recent studies have pointed out several miRNAs as key regulators of the Ca^{2+} current (I_{Ca}) and I_{K1} in AF cardiomyocytes.[13,14] To date, a pair of miRNAs

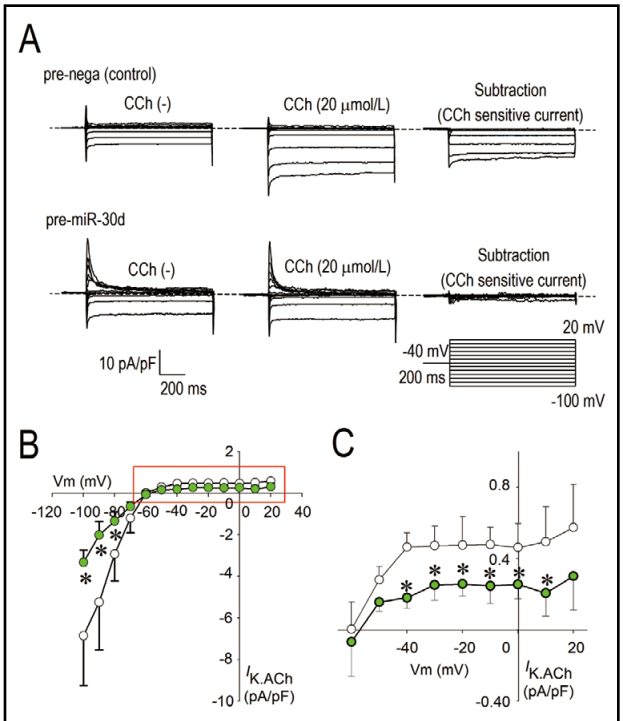

Figure 5. Effects of miR-30d (pre-miR-30d) overexpression on the acetylcholine-sensitive inward-rectifier K^+ current ($I_{K.ACh}$). (**A**) Representative $I_{K.ACh}$ traces recorded in neonatal rat cardiomyocytes after transfection with negative control (pre-nega) or pre-miR-30d for 24 h. I_{KACh} was obtained by subtraction of the trace at basal current from the trace in the presence of carbachol (CCh; 20 μmol/L), which is defined as the acetylcholine-sensitive current component, $I_{K.ACh}$. (**B**,**C**) Current (I)-voltage (V) relationship for $I_{K.ACh}$: open circles represent pre-negative control-transfected myocytes (n=8) and green circles are for pre-miR-30d-transfected myocytes (n=5). The red-framed rectangle for the outward component of $I_{K.ACh}$ is shown in an enlarged scale in (**C**). *P<0.05 vs. negative control-transfected cardiomyocytes.

(miR-21 and miR-328) have been identified in the human AF atrium as negative modulators of Cav1.2 (*CACNA1C*), although we could not detect changes of miR-328 (fold change, 1.09) and miR-21 (fold change, 1.07) in miRNA microarray data. Quantitative RT-PCR analysis could not detect significant change in miR-328 and miR-21 either (**Figure S6**). miR-21 is highly associated with heart failure,[23] which may indicate that miR-21 can be associated with AF etiologically linked to heart failure. While miR-328 is highly upregulated (atrial myocytes) in a new onset of AF in an animal experiment,[13] and downregulated (circulating blood) in patients with persistent AF,[24] this suggests that miR-328 could be affected by the time-course of AF progression. The present study demonstrates that the mRNA and protein levels for Cav1.2 were suppressed in atrial myocytes with persistent AF (**Figure 3**), which is consistent with previous report findings.[13,14] Given these and our study findings, the mechanisms of $I_{Ca.L}$ downregulation during AF may include transcriptional changes caused by NFAT modulation,[6] as well as post-transcriptional ones, possibly mediated by several miRNAs indirectly affecting the Cav1.2 gene, and/or in a fashion depending on the progress of the disease.

Unlike Cav1.2, most studies investigating the post-transcriptional regulation of Kir2.1 (*KCNJ2*) are consistently demonstrating a role of miR-1 and miR-26 in AF cardiomyocytes.[7,15]

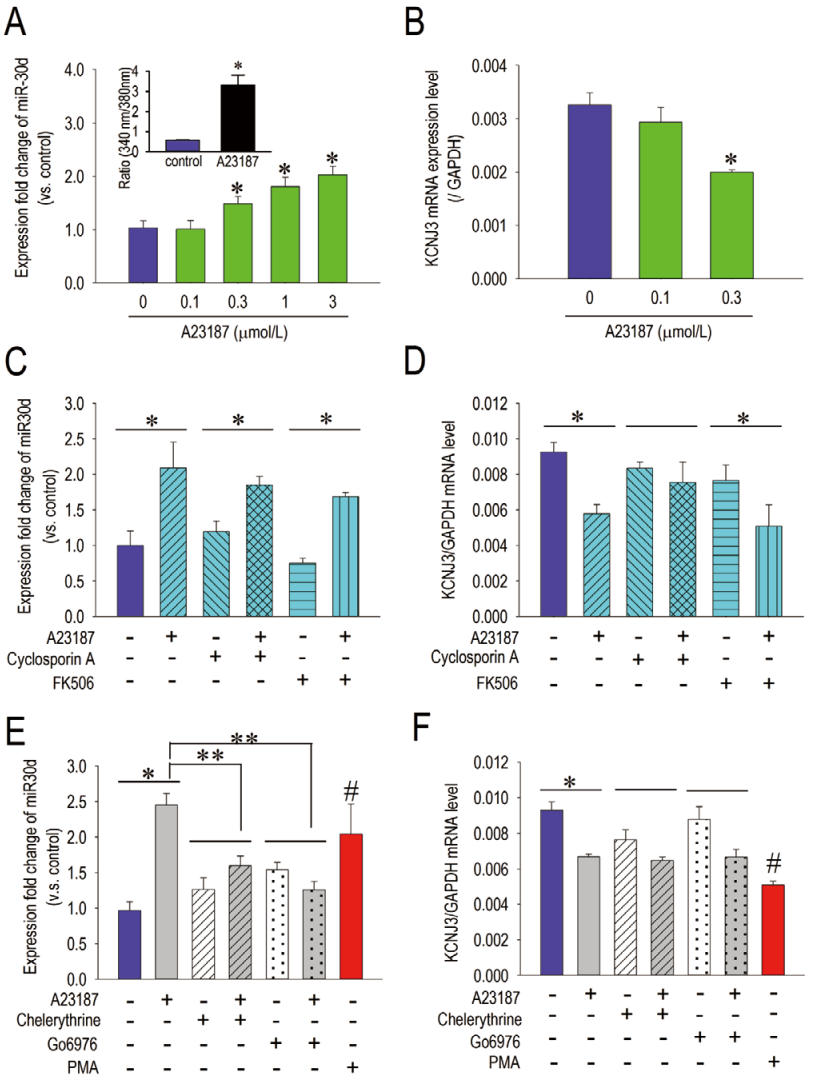

Figure 6. Modulation of the expression of miR-30d and *KCNJ3* by intracellular Ca^{2+} and protein kinase C (PKC) activation. (**A**) Effects of intracellular Ca^{2+} overloading, by application of the Ca^{2+} ionophore A23187, on miR-30d expression. Cardiomyocytes were incubated with A23187 at concentrations of 0.1–3 μmol/L for 24 h. (**B**) Effects of A23187 on *KCNJ3* mRNA expression. (**C**,**D**) Effects of Ca^{2+}-nuclear factor of activated T-cells (NFAT) signal inhibition on the expression of miR-30d (**C**) and *KCNJ3* (**D**). Cardiomyocytes were incubated with A23187 (0.3 μmol/L) in the presence or absence of cyclosporin A (1 μmol/L) or FK506 (1 nmol/L) for 24 h. (**E**,**F**) Effects of Ca^{2+}-PKC signal inhibition on the expression of miR-30d (**E**) and *KCNJ3* (**F**). Cardiomyocytes were incubated with A23187 (0.3 μmol/L) in the presence or absence of chelerythrine (1 μmol/L) or Gö6976 (10 nmol/L) for 24 h. Red bars represent treatment with the PKC activator, phorbol 12-myristate 13-acetate (PMA; 1 μmol/L), for 24 h. Data are expressed as the mean±SE (n=8). *,#P<0.05 vs. control (A23187 (–)). **P<0.05 vs. A23187-treated cardiomyocytes.

In agreement with previous studies, an increase of the inward-rectified K^+ channel (*KCNJ2*) in AF cardiomyocytes was confirmed in the present study (**Figure 3**). Of note, an in silico approach assumes the binding not only of miR-1 and miR-26, but also of other miRNAs to the 3´-UTR of the *KCNJ2* gene, which suggests the necessity of a functional assay to finally determine the mechanisms of miRNA-dependent remodeling of *KCNJ2* in AF.

Aging, in addition to its impact on many different miRNAs expressions,[25] is one of the main causes of development of AF. Moreover, many factors and diseases, including hypertension,[26] diabetes,[27] chronic heart failure,[28] inflammation disease,[29] and history of steroid therapy,[30] can possibly affect the level of miRNAs as well as several genes independently.[21] This is probably because AF is a complex disease with a multifactorial etiology.[31–34] In order to avoid a biased sampling from AF patients, we carefully selected patients in this study that strictly represent samples to elucidate AF-specific changes in the miRNAs of the human atrium. Importantly, electrical remodeling in atrial cardiomyocytes with chronic AF is distinct from those with paroxysmal AF.[17] We adopted human atrial tissues only from men with no recorded AF history and from men with an AF history of 6 months or longer, whose age ranged from 60 to 79 years. Accordingly, to the best of our knowledge, miR-30d was identified for the first time as one of the most abundantly expressed and significantly upregulated miRNA by this comprehensive analysis of miRNA expression profiles in the human atrium with persistent AF.

Regulation of miR-30d in Cardiomyocytes With AF

The miR-30 family is one of the most highly expressed miRNAs in the heart.[12,35] Among 355 of 1,205 human miRNAs being expressed and assumed functioning in the heart (**Figure S1A**), miR-30d is the 6th most abundantly expressed miRNA (**Table S3**), which indicates that it could be a crucial regulator of cardiomyocytes in the physiological and/or pathophysiological condition. MiR-30d has also been observed in a variety of organs, where it is responsible for apoptosis,[36] the cell cycle,[37] and differentiation.[38] Although some studies have revealed the action of miR-30d in the myocardium, there is no evidence indicating its role for the post-transcriptional regulation of ion channels.

The majority of miRNA genes are located in intergenic regions (indicating that they form independent transcription units)[9] where miR-30d belongs to the intergenic miRNA gene

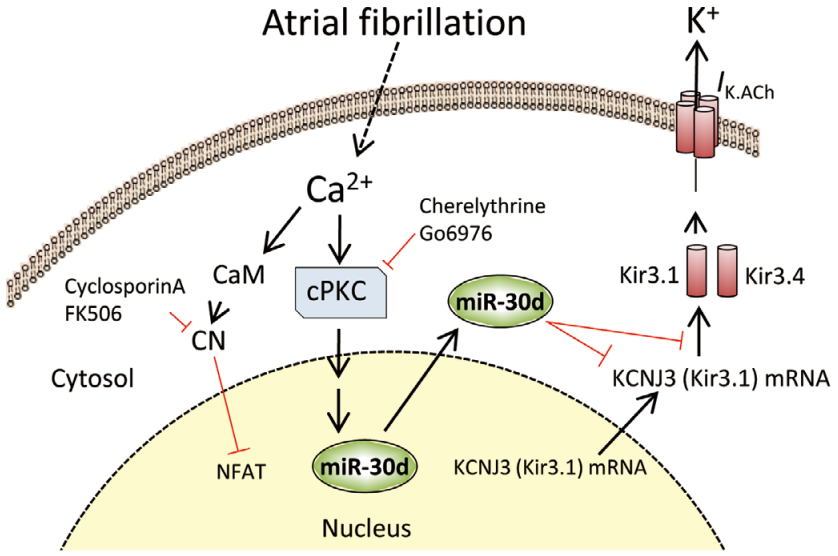

Figure 7. Schematic representation of the inward-rectifier K^+ current ($I_{K.ACh}$) remodeling underlying Ca^{2+} overload in cardiomyocytes with atrial fibrillation. The elevation of miR-30d expression caused by activation of $[Ca^{2+}]_i$-dependent conventional protein kinase C (cPKC) suppresses its target gene, *KCNJ3*, at both the mRNA and protein levels, causing a reduction of $I_{K.ACh}$.

group.[39] In this case of gene groups, little is known about the regulatory mechanism for the responsible transcription factors. A number of possible causes for atrial electrical remodeling have been proposed; these include a shift in autonomic tone, mechanoelectrical feedback, or a direct effect of tachycardia on potassium channels. Like myocardial ischemia, sustained rapid atrial rates and a persistent atrial tachyarrhythmia might overwhelm exchange pumps as well as other homeostatic mechanisms, resulting in cytosolic calcium overload. In this context, intracellular calcium overload was examined in order to simulate the pathological condition of cardiomyocytes with AF. Exposure of myocytes to the Ca^{2+} ionophore A23187 induced the upregulation of miR-30d in a dose-dependent manner, which concomitantly decreased the *KCNJ3* mRNA levels, which was blocked by PKC inhibition (**Figure 6**). With regard to the role of the PKC pathway in the regulation of $I_{K.ACh}$, Markary et al[40] demonstrated that activation of PKC inhibited $I_{K.ACh}$ as a short-term effect in atrial myocytes, whereas Puglisi et al[41] demonstrated that activation of PKC suppressed the *KCNJ3* gene as a long-term effect, where the underlying mechanism was uncovered. Taken these observations into consideration, we attribute the long-term augmentation of $I_{K.ACh}$ by PKC, at least in part, to the action of miR-30d. Cellular Ca^{2+} overload plays a crucial role in myocardial hypertrophy,[12] apoptosis,[39] and ion channel remodeling.[10] MiR-30 family members are highly expressed in cardiomyocytes, not only in persistent AF but also in hypertrophied[12] or apoptotic tissues.[39] Given all these results, it is suggested that miR-30d is a pivotal molecule in cardiomyocytes, responding to cellular Ca^{2+} overload in the pathological condition of the heart. The roles of NFAT signals for miR-30d-Kir3.1 pathways are yet to be clarified. We found that application of cyclosporin A to block NFAT signals induced a reduction of *KCNJ3* mRNA expression independently of the action of miR-30d (**Figures 6D**,**S5**), raising the possibility that the inhibition of calcineurin/NFAT activity may lead to the transcriptional regulation of *KCNJ3*. The PKC-dependent transcriptional regulation of *KCNJ3* may also explain the results, because PKC blockade did not change the expression of miR-30d, but it did reduce the expression of *KCNJ3*. Investigations to identify the signals for *KCNJ3* transcription will also be needed.

Remodeling of $I_{K.ACh}$ in Cardiomyocytes With Persistent AF

The acetylcholine-sensitive inward-rectifier K^+ current ($I_{K.ACh}$) is one of the important determinants for atrial excitability. Sustained AF causes a shortening of the APD in atrial cardiomyocytes, leading to a shortening of the atrial refractory period.[3,5,8] Despite the relevant electrophysiological changes in atrial excitability, the molecular mechanism for $I_{K.ACh}$ remodeling is poorly understood. Remodeling of $I_{K.ACh}$ in AF cardiomyocytes still remains controversial.[4,42,43] Recent studies revealed a reduction of mRNA and/or protein of Kir3.1 in human AF atrial myocytes.[4,43] In contrast, an increase of $I_{K.ACh}$ was observed by Bosch et al[42] in isolated human cardiomyocytes with AF. Interestingly, but perhaps not surprisingly, a recent study identified the long-term upregulation of $I_{K.ACh}$ in AF atrial cardiomyocytes that was not accompanied by an upregulation of Kir3.1/Kir3.4 proteins;[40,43] constitutively active $I_{K.ACh}$ is predominantly observed in the remodeled atrium from patients with permanent AF.[3,44] Among the 2 homologous proteins (Kir3.1 and Kir3.4) comprising cardiac $I_{K.ACh}$, we observed a downregulation of Kir3.1 (*KCNJ3*) mRNA and protein in myocytes with persistent AF, whereas the level of Kir3.4 (*KCNJ5*) mRNA was unchanged (**Figure 3B**). Because the interactive binding sites of 39 distinct miRNAs (including miR-30d) were absent in the 3´-UTR of the Kir3.4 (*KCNJ5*) gene, we did not further investigate Kir3.4 as a target of post-transcriptional remodeling in AF.

Study Limitations

Although the present study provides novel evidence that miR-30d is a candidate of post-transcriptional mediators for ion channel remodeling, caused relatively solely by persistent AF, we could not exclude other mechanisms. Also, a characteristic of miRNA microarray from human samples may only represent features of persistent AF-dependent remodeling but not of paroxysmal AF-dependent remodeling. Despite the efforts of Schoots et al,[45] the cardiomyocytes-specific transcriptional regulation of the gene coding for human Kir3.1 remains

unknown. Because of this reason, we were unable to study the possible transcriptional mechanism for Kir3.1 remodeling in AF. We could not observe the development or inducibility of AF by in vivo administration of exogenous miR-30d oligonucleotides. Because the structure of miRNAs and/or their interaction with the target genes are species dependent, careful in vivo animal experiments to identify the specific roles of miRNAs would be needed. As a case-in-point, the interactive binding site of miR-30d to the human 3´-UTR for Kir3.1 is conserved in the rat but not in the mouse. Moreover, we could not examine the role of miR-30d in human atrial cardiomyocytes because of ethical limitations. Experiments with human-induced pluripotent stem cells as a substitute for human cardiomyocytes would also be needed in the future, before drawing the final conclusions.

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Disclosures

None.

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Supplementary Files

Supplementary File 1

Methods

Table S1. Baseline characteristics of patients with persistent AF and matched control patients in NSR

Table S2. Sequence of oligonucleotides used as real-time PCR primers

Table S3. Representative miRNAs differentially expressed between NSR and AF in human cardiomyocytes

Table S4. Prediction of the position of miR-30d binding regions with its target sites in the 3´-UTRs of human CACNA1C and KCNJ3

Figure S1. Analysis of microRNA (miRNA) microarray data.

Figure S2. Detection of *KCNJ3* mRNA in adult (10 weeks old) and neonatal (0–1 days old) rat hearts.

Figure S3. Molecular structure of miR-30 family members and effect of miR-30d knockdown on the level of miR-30 family members in cardiomyocytes.

Figure S4. Effects of Ca^{2+} overload, by the Ca^{2+} ionophore A23187, on the expression of miR-30b and miR-30d in rat cardiomyocytes.

Figure S5. Effects of Ca^{2+}-nuclear factor of activated T-cells (NFAT) signal inhibition on *KCNJ3* mRNA expression in rat cardiomyocytes.

Figure S6. Quantitative RT-PCR performed on miR-21 and miR-328 in atrial cardiomyocytes from all 33 patients (n=19 for normal sinus rhythm (NSR) and n=14 for atrial fibrillation (AF)).

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-15-1276