MiR-647 Targeting PRKCA to Regulate the Activation of Human Atrial Fibroblasts Through TGFβ1/Smad Pathway

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Research Article

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Abstract

Background

Atrial remodeling is the main pathogenesis of atrial fibrillation (AF). Interstitial fibrosis is an important part of atrial remodeling, mainly involving the activation of atrial fibroblasts. Nowadays, studies have focused on miRNAs in regulating fibroblasts in AF related atrial fibrosis, however, the molecular mechanism of this pathological process is not fully understood.

Methods

Cell experiments were carried out to investigate the mechanism of miR-647 targeting PRKCA gene in regulating atrial fibrosis of AF. CCK-8, transwell, immunofluorescence assays were applied to observe the proliferation, migration and the phenotypic transformation of the human atrial fibroblasts. qRT-PCR and WB were used to detect the collagen production, expression levels of α-SMA and TGFβ1/Smad pathway.

Results

The results of CCK-8, transwell and immunofluorescence assays showed that miR-647 inhibited the proliferation, migration and the fluorescence intensity of α-SMA of the human atrial fibroblasts. QRT-PCR and WB showed that miR-647 could significantly attenuate the expression levels of fibrosis related Collagen I and III, α-SMA and TGFβ1/Smad pathway by targeting PRKCA.

Conclusion

MiR-647 targeting PRKCA gene regulates the activation of human atrial fibroblasts through the TGFβ1/Smad pathway.

Introduction

Atrial fibrillation (AF) has quite a substantial incidence among all pathological arrhythmias, and its morbidity is increasing worldwide. The mortality of patients with AF is twice as much as those without AF, which will undoubtedly bring a significant socioeconomic burden.[1, 2] Therefore, it is of great importance to explore the pathogenesis of AF and search for effective therapeutic targets.

The pathogenesis of AF is complex and mainly contains atrial electrical and structural remodeling, which interacts with each other to promote the occurrence and evolution of AF.[3] The differentiation of atrial fibroblasts into myofibroblasts, which is stimulated by fibrogenic factors such as angiotensin II, platelet-derived growth factor and transforming growth factor-beta 1 (TGFβ1), is an important pathophysiological mechanism during the atrial structural remodeling.[4, 5] Previous studies reported that the classic

TGFβ1/Smad signaling pathway is mostly involved in fibroblasts activation, to promote the secretion and deposition of the extracellular matrix (ECM), and ultimately leading to atrial fibrosis.[6, 7]

MicroRNAs (miRNAs) are endogenous non-coding small RNAs that play an important role in post-transcriptional regulation of the down-stream target genes. It is reported that miRNAs are involved in regulating cardiac remodeling, especially in the process of AF related atrial fibrosis.[8] For instance, MiR-133 and miR-590 regulate atrial fibrosis via targeting TGFβ1 and TGFβRII;[9] MiR-26 inhibits KCNJ2 to decrease the deposition of ECM;[10] MiR-29 targets collagen I and III directly to reduce atrial fibrosis;[11] Inhibition of miR-21 could up-regulate the expression of SPRY1 to reduce AF burden.[12] The miR-647 has been reported to participate in the pathophysiologic process of many malignant cancers.[13–15] However, the role of miR-647 in heart diseases including AF related atrial fibrosis is not fully understood.

Protein kinase Calpha, encoded by PRKCA gene, takes part in the pathogenesis of tumor, cardiovascular system and nervous system diseases. Recent researches discovered that up-regulation of PRKCA could increase the fibrosis of renal and liver.[16, 17] Additionally, PRKCA increases the vulnerability to AF by dysregulation of calcium signaling, suggesting that PRKCA may be involved in the occurrence and maintenance of AF.[18]

In our previous studies, bioinformatics analysis was used to analyze the differentially expressed genes in atrial tissues of sinus rate (SR) and AF patients. MiR-647 and its predicted target gene PRKCA were screened out, which may be involved in AF. MiR-647 was detected as down-regulated and PRKCA was up-regulated in the atriums of AF patients. Therefore, we hypothesized that miR-647 may target PRKCA, then regulate atrial fibrosis via the TGFβ1/Smad pathway. To address this, we explored the interaction between miR-647 and PRKCA in human atrial fibroblasts, and investigated the role of the miR-647/PRKCA axis in regulating atrial fibrosis.

Methods

Cell culture and identification

Primary human atrial fibroblasts were bought from ScienCell (Cat. #6320, San Diego, CA, USA). The cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, ScienCell, Cat. #0025), 1% Fibroblast Growth Supplement-2 (ScienCell, Cat. #2382) and 1% penicillin/streptomycin solution (ScienCell, Cat. #0503) in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed every 3 days. The cells at passages 3–5 were used in the following experiments.

For atrial fibroblasts identification, the cells were fixed in 4% paraformaldehyde for 5 min and washed with phosphate buffer solution (PBS) for three times. Then, the cells were blocked with 1% Triton×100 for 30 min at room temperature and washed with PBS. Blocked with 5% bovine serum albumin (BSA) for 1 h, the cells were then incubated with anti-vimentin (CST, MA, USA) overnight at 4°C. On the next day, washed

the cells with PBS and incubated with the secondary antibody IgG (CST) for 1 h. The cells were then incubated with DAPI (Beyotime, Shanghai, CN) at room temperature for 5 min and observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Cell transfection

MiR-647 and PRKCA shRNA or control shRNA lentiviral particles were designed and synthesized by Genechem Co., Ltd (Shanghai, CN). Overexpression lentiviral particles of hsa-miR-647, hsa-PRKCA and control were also synthesized by GeneChem.

Human atrial fibroblasts were seeded on 6-well plates 24 h prior to transfection. The cells were then transfected with individual shRNAs or overexpression lentiviral particles using polybrene according to the manufacturer's instructions (MOI = 50). The cells were cultured for an additional 12–16 h and replaced with fresh medium. After transfection for 3–5 days, the green fluorescence could be observed under a fluorescence microscope (Nikon, Tokyo, Japan).

RNA isolation and quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA of human atrial fibroblasts was isolated using the Total RNA Extraction Kit (TaKaRa, Japan). cDNA was synthesized using the Reverse Transcription System Kit (TaKaRa) according to the manufacturer's instructions. QRT-PCR was conducted in optical 96-well plates using SYBR Green PCR Mix (TaKaRa) in a Step One Real-Time PCR System (Bio-Rad, CA, USA) according to the manufacturer's instructions. The relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Expression of miR-647 was normalized to U6 small nuclear RNA. Expression of PRKCA, Collagen I, Collagen III, Smad2, Smad3, α-SMA, TGFβ1 and TGFβRII was normalized to GAPDH. All primer sequences are listed in Table 1.

Table 1
Primers for qRT-PCR

Genes	Primer sequence (5' to 3')	
GAPDH	F	TCAAGAAGGTGGTGAAGCAGG
	R	GCGTCAAAGGTGGAGGAGTG
miR-647	F	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAAGGAAG
	R	ACACTCCAGCTGGGGTGGCTGCACTCACT
U6	F	CTCGCTTCGGCAGCACA
	R	AACGCTTCACGAATTTGCGT
URP	R	TGGTGTCGTGGAGTCG
PRKCA	F	CTGTGGGTCACTGCTCTATGGA
	R	GGATGGTTTTGGTTTTTTGCTT
TGFβ1	F	CAGCAACAATTCCTGGCGATACCT
	R	CGCTAAGGCGAAAGCCCTCAAT
TGFβRII	F	AAAAAAACAGTGGGAAGACC
	R	AACTCCATAAATACGGGCAT
Smad2	F	GTCTCCAGGTATCCCATCG
	R	TTAGGATCTCGGTGTGTCGG
Smad3	F	ACGACTACAGCCATTCCATCCC
	R	CATCTGGTGGTCACTGGTTTCTC
Collagen I	F	AAAGATGGACTCAACGGTCTC
	R	ACCAGCTTCACCAGGAGATC
Collagen III	F	AAAAGGGGAGCTGGCTACTT
	R	GAATTTCTGGGTTGGGGCAG
α-SMA	F	TCATGGTCGGTATGGGTCAG
	R	CGTTGTAGAAGGTGTGGTGC

Protein extraction and western blotting (WB) analysis

Proteins of human atrial fibroblasts were extracted using RIPA buffer containing protease and phosphatase inhibitors (Beyotime, Shanghai, CN). The BCA protein assay kit (ThermoFisher Scientific, MA, USA) was used to detect the protein lysate concentrations according to the manufacturer's protocols.

For WB, 20 μg protein in each group was separated by running the sample on a NuPage 4–12% Bis-Tris gel (Sangon, Shanghai, CN). The protein was transferred to PVDF membranes (Millipore Corp. Billerica, MA, USA) and blocked for 1 h with 5%BSA in Tris-buffered saline with Tween 20 (TBST) buffer at 4°C, then incubated overnight with antibodies targeting β-actin (Boster, 1:500), PRKCA (Abcam, 1:5000), Collagen I (Abcam, 1:2000), Collagen III (Abcam, 1:5000), α-SMA (CST, 1:1000), Smad2 (Affinity, 1:1000), p-Smad2 (CST, 1:1000), Smad3 (Abcam, 1:1000), p-Smad3 (CST, 1:1000), TGFβ1 (Santa Cruz, 1:300), TGFβRII (Santa Cruz, 1:300) in TBST with 5% BSA at 4°C. Membranes were washed for 5 times with TBST for 5 min each time and incubated with the secondary goat anti-rabbit and goat anti-mouse IgG (Boster, 1:5000) in TBST buffer with 5% BSA at room temperature for 1 h. The blots were then washed 5 times for 5 min each time in TBST, the antibody interactions and the grayscale intensities were detected with the ECL-PLUS/Kit (Vazyme, Nanjing, CN). The experiments were repeated at least three times.

CCK-8 cell proliferation assay

The proliferation of the human atrial fibroblasts was measured by CCK-8 kit (Beyotime, Shanghai, CN) according to the manufacturer's protocols. In brief, the cells at passages 3–5 were seeded in 96-well plates at a density of 2×10^3 per well. Then, the cells were transfected with the lentiviral particles and treated with 5ng/mL TGFβ1 (R&D Systems, MN, USA) for 48 h. Then, the cells in each well were incubated with 10 μL CCK-8 solution at 37°C for 2 h. The optical density (OD) values at 450 nm were recorded on a microplate reader (BioTek, VT, USA).

Transwell migration assay

The human atrial fibroblasts (1×10^5 cells) were seeded onto the transwell chambers in 24-well plates with 800 μL DMEM containing 10% FBS. After 24 h incubation, media within the transwell chambers were carefully removed and washed the chambers with PBS. Cells that did not migrate across the transwell membrane were removed by gently by a cotton swab. Then, the cells were fixed with 10% methanol for 30 min and stained with crystal violet (Sigma-Aldrich) for 20 min. The migrated cells were observed under the inverted phase contrast microscope (Nikon, Japan).

Immunofluorescence staining assay

Washed the glass slides of human atrial fibroblasts with PBS for 3 times, fixed the cells in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for another 20 min at room temperature. Then, blocked the cells with goat serum for 30 min and incubated with the primary antibody against α-SMA (CST, MA, USA) at 4°C overnight. The next day, washed the cells with PBS and incubated with fluorescently conjugated secondary goat anti-mouse antibody (Boster) and DAPI (Beyotime) at room temperature in the dark. Sealed the slides and observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Dual luciferase reporter gene assay

The 3'-UTR of PRKCA containing the predicted wild-type (wt) binding sites of miR-647 or mutated miR-647 binding sites (mu) were amplified by PCR and inserted into a pGL3 basic vector (Promega, WI, USA)

to generate PRKCA-wt, PRKCA-mu. Co-transfected with the constructed luciferase reporter vectors, miR-647 mimic or miR-negative control (mimic NC) into human atrial fibroblasts. At 48 h post-transfection, luciferase activities were detected by a dual-luciferase reporter assay kit (Promega).

Statistical analysis

Statistical analyses were performed using Graphpad Prism 7 software (CA, USA). The Student's t-test was employed to analyze differences between the two groups. One-way ANOVA was used for the comparison between groups. $P < 0.05$ was considered as statistically significant. All experiments were repeated at least three times.

Results

Identification of human atrial fibroblasts

The human atrial fibroblasts were vimentin-positive, vWF- and α-SMA-negative. For identification, the cells were stained with anti-vimentin in green and the nucleus were stained with DAPI in blue, to confirm the cells were human atrial fibroblasts (Fig. 1).

miR-647 directly regulated PRKCA expression

Our previous bioinformatics analysis revealed that miR-647 carried putative binding sites of PRKCA (Fig. 2A). To test whether miR-647 directly targets PRKCA in atrial fibroblasts, PRKCA-wt, PRKCA-mu, miR-647 mimic or mimic NC were co-transfected into the human atrial fibroblasts for an additional 48 h. The dual luciferase activity assay showed that compared with the mimic NC group, the miR-647 mimic caused a remarkable decrease in luciferase activity in PRKCA-wt reporter, whereas had no evident effect on luciferase activity in PRKCA-mu reporter (Fig. 2B).

To further explore the effect of miR-647 on PRKCA, the mimic NC, miR-647 mimic, inhibitor NC, and miR-647 inhibitor were transfected into the TGFβ1-induced human atrial fibroblasts respectively (Supplementary Fig. 1). The qRT-PCR was applied to confirm the overexpression and knockdown efficiency of miR-647 in human atrial fibroblasts (Fig. 2C). The results of qRT-PCR and WB revealed that the expression of PRKCA significantly decreased in the cells transfected with miR-647 mimic compared with mimic NC ($P < 0.05$), and the expression of PRKCA significantly increased in the cells transfected with miR-647 inhibitor compared to inhibitor NC ($P < 0.05$) (Fig. 2D, 2E). Taken together, these results indicated that miR-647 directly regulated PRKCA expression in human atrial fibroblasts.

miR-647 expression inhibited the proliferation, migration, transformation, collagen production, and TGF-β1/Smad signaling activation of TGFβ1-induced human atrial fibroblasts

To explore the effects of miR-647 on the proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation of the TGFβ1-induced human atrial fibroblasts, the mimic NC, miR-647 mimic, inhibitor NC, and miR-647 inhibitor were transfected into the TGFβ1-induced human atrial

fibroblasts as previously described. After stimulating by TGFβ1, the proliferation of the human atrial fibroblasts was significantly promoted, which was then blocked by miR-647 overexpression, whereas promoted by miR-647 knockdown ($P < 0.05$) (Fig. 2F, Supplementary Table 1). The cell migration was obviously facilitated after TGFβ1 stimulation, and was reduced by miR-647 mimic, but accelerated by miR-647 inhibitor ($P < 0.05$) (Fig. 2G, Supplementary Fig. 2). Transformation of fibroblasts into myofibroblasts is a hallmark of fibrosis, and α-SMA is a representative marker of this process. Therefore, we also detected the fluorescence intensity and expression of α-SMA to confirm the effect of miR-647 on the transformation of human atrial fibroblasts. TGFβ1 stimulation augmented the fluorescence intensity and expression of α-SMA in the human atrial fibroblasts, miR-647 overexpression significantly reduced the fluorescence intensity and α-SMA expression, which were increased by miR-647 knockdown ($P < 0.05$) (Fig. 2H, Supplementary Fig. 3, 4D, 4E).

Additionally, miR-647 mimic evidently down-regulated the TGFβ1-induced expression of collagen I, collagen III, TGFβ1/Smad signaling-related proteins, whereas miR-647 inhibitor exerted the opposite effects ($P < 0.05$) (Fig. 2I, 2J, 2K, Supplementary Fig. 4A, 4B, 4C). These results indicated that the miR-647 overexpression attenuated TGFβ1-induced human atrial fibroblasts proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation, which were facilitated by miR-647 knockdown.

PRKCA expression promoted the proliferation, migration, transformation, collagen production, and TGF-β1/Smad signaling activation of TGFβ1-induced human atrial fibroblasts

Next, we investigated whether PRKCA was responsible for the proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation of the TGFβ1-induced human atrial fibroblasts. The lentiviral particles of vector, PRKCA, si-ctrl, and si-PRKCA were transfected into the TGFβ1-induced human atrial fibroblasts independently (Supplementary Fig. 5). The results of qRT-PCR and WB revealed the overexpression and knockdown efficiency of PRKCA in human atrial fibroblasts (Fig. 3A, 3B). The proliferation of the human atrial fibroblasts was significantly accelerated by PRKCA overexpression, whereas suppressed by PRKCA knockdown ($P < 0.05$) (Fig. 3C, Supplementary Table 2). The cell migration was obviously facilitated by PRKCA overexpression, but attenuated by PRKCA knockdown ($P < 0.05$) (Fig. 3D, Supplementary Fig. 6). PRKCA overexpression significantly augmented the fluorescence intensity and α-SMA expression of TGFβ1-induced human atrial fibroblasts, which were inhibited by PRKCA knockdown ($P < 0.05$) (Fig. 3E, Supplementary Fig. 7, 8D, 8E).

Additionally, PRKCA overexpression obviously increased the TGFβ1-induced expression of collagen I, collagen III, TGFβ1/Smad signaling-related proteins, nevertheless, PRKCA knockdown reversed the effects ($P < 0.05$) (Fig. 3F, 3G, 3H, Supplementary Fig. 8A, 8B, 8C). These results demonstrated that PRKCA overexpression facilitated TGFβ1-induced fibroblasts proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation, which were reversed by PRKCA knockdown.

miR-647 inhibited the TGFβ1-induced human atrial fibroblasts proliferation, migration, transformation, collagen production, and TGF-β1/Smad signaling activation by targeting PRKCA

To further verify whether PRKCA was responsible for miR-647-mediated regulation of fibroblasts proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation under TGFβ1 stimulation. The lentiviral particles of miR-647, vector, and PRKCA were co-transfected into the TGFβ1-induced human atrial fibroblasts (Supplementary Fig. 9). The results of qRT-PCR and WB showed the transfection efficiency of miR-647 and PRKCA in human atrial fibroblasts (Fig. 4A, 4B, 4C). The miR-647 inhibited the proliferation of the human atrial fibroblasts, which was promoted when PRKCA was overexpressed ($P < 0.05$) (Fig. 4D, Supplementary Table 3). Meanwhile, PRKCA overexpression reversed the miR-647-inhibited migration of the cells ($P < 0.05$) (Fig. 4E, Supplementary Fig. 10). PRKCA overexpression also significantly reversed the miR-647-mediated attenuation of fluorescence intensity and α-SMA expression of TGFβ1-induced human atrial fibroblasts ($P < 0.05$) (Fig. 4F, Supplementary Fig. 11, 12D, 12E).

Moreover, PRKCA overexpression significantly increased the expression of collagen I, collagen III, TGFβ1/Smad signaling-related proteins in TGFβ1-induced human atrial fibroblasts, which were inhibited by miR-647 overexpression ($P < 0.05$) (Fig. 4G, 4H, 4I, Supplementary Fig. 12A, 12B, 12C). These data verified that PRKCA was responsible for miR-647-mediated regulation of fibroblasts proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation under TGFβ1 stimulation.

Discussion

Atrial remodeling includes electrical remodeling and structural remodeling, is the main cause AF. Atrial fibrosis is an important component of structural remodeling, which is associated the occurrence, development, resistance to therapy of AF. The abnormal proliferation, migration, transformation of atrial fibroblasts into mypfibroblasts and excessive deposition of ECM are the major pathological changes during this process.[3, 4] Here, in TGFβ1-treated human atrial fibroblasts, miR-647 overexpression could significantly inhibit the proliferation, migration and transformation of atrial fibroblasts, as well as reduce the expression levels of collagen I, collagen III, α-SMA and TGFβ1/Smad signaling pathway by targeting PRKCA. These data demonstrated that miR-647 regulates the activation of human atrial fibroblasts by targeting PRKCA, indicating the anti-fibrotic role of miR-647 in AF (Fig. 5).

MiRNAs are important molecules in post-transcriptional regulation, which play a key role in the pathogenesis, diagnosis and treatment of various diseases. Previous studies have shown that miRNAs are involved in regulating all types of cardiac remodeling paradigms, especially in AF related fibrotic responses. Most AF related miRNAs are down-regulated in the atrial tissues, including miR-1, miR-26, miR-29, miR-133 and miR-590.[9–11, 19] In addition, some up-regulated miRNAs are also involved in the pathogenesis of AF containing miR-21, miR-23b, miR-199b and miR-328.[12, 20–23] These abnormal expressed miRNAs activate AF related signaling pathways and promote atrial fibrosis. In our previous research, miR-647 has been screened out by bioinformatics and detected to be down-regulated in the atrial tissues of AF patients, which has been reported only in the field of cancer research before. Ma et al. discovered that decreased expression of serum miR-647 is associated with poor prognosis in gastric

cancer.[13] Qin et al. reported that miR-647 inhibited glioma cell proliferation, colony formation and invasion by regulating HOXA9.[14] Xu et al. found that miR-647 promotes proliferation and migration of ox-LDL-treated vascular smooth muscle cells through regulating PTEN/PI3K/AKT pathway.[15] Our study firstly proved that miR-647 participated in the abnormal proliferation, migration, transformation of atrial fibroblasts into myofibroblasts and excessive deposition of ECM by targeting PRKCA gene, which is an important pathophysiological mechanism of AF related fibrosis.

At present, researchers are making efforts to study miRNA targets for therapeutic purposes. For example, antisense oligonucleotide inhibitor of miR-122 has entered phase II clinical trials for the treatment of hepatitis C.[24] Liposome encapsulated miR-34 mimics is now in phase I clinical trials in treating cancer.[25] However, the treatment of AF based on miRNAs still remains challenge, which is related to the unique characteristics of AF, the multiple target points and the off-target effects of miRNA. For instance, in heart failure caused AF models, at least 8 fibrosis related miRNAs altered, making specific targeting difficult.[26] Nevertheless, there are still some miRNAs that may serve as promising therapeutic targets for AF such as miR-21, which is upregulated in AF models and takes part in multiple fibrotic pathways;[12] miR-26, which is involved in AF related atrial remodeling.[10] Whether miR-647 could work as a potential target for AF treatment needs further investigations. This field is still in its infancy and shows both prospects and barriers to application.

Protein kinase Calpha (PKCα), encoded by PRKCA gene, is a key enzyme in G protein signaling pathway. Previous studies reported that PKCα takes part in the pathogenesis of tumor, cardiovascular system and nervous system diseases, plays an important role in intracellular signal transduction process and is closely related to the expression and function of various ion channels.[27, 28] Luzum et al. studied the relationship between the heart disease and PRKCA mutants and found that the haplotype mutation carrying rs9909004 could affect the expression level of PRKCA in the heart, and was associated with the occurrence and prognosis of heart failure patients.[29] Other studies have shown that PRKCA was up-regulated in atrial hypertrophy, and its mutation was closely related to the QRS interval, suggesting that PRKCA may be involved in both cardiac structural remodeling and electrical remodeling.[30, 31] PRKCA has also been reported to be involved in the fibrosis process of liver, kidney and other organs.[16, 17] Our results showed that PRKCA is the target gene of miR-647, participate in miR-647 regulated process of atrial fibrosis, indicating that PRKCA is an important gene in the pathogenesis of AF.

TGFβ1/Smad signaling pathway plays an important role in the fibrosis process of liver, lung, kidney, skin and myocardial tissues.[32] TGFβ1 is secreted as a potentially high molecular weight complex (over 200 kDa), consisting of a mature and biologically active TGFβ1 (25 kDa) and a predomain of potentially binding polypeptide. TGFβ1 binds to the extracellular TGF-binding proteins to form the TGFβ complex, which is activated by proteolysis and binds to TGFβRII. Smad2/3, the receptor-activated Smad complex, binds to TGFβRII and phosphorylates itself to the activated p-Smad2/3. P-Smad2/3 aggregates in the nucleus to regulate transcriptional responses.[33, 34] Therefore, TGFβ1 stimulates collagen transcription and ECM deposition in fibroblasts and promotes the transformation of fibroblasts into myofibroblasts through Smad signaling.

TGFβ1/Smad pathway is regulated by many upstream genes. PRKCA has not been reported to participate in atrial fibrosis by regulating TGFβ1/Smad pathway. This study verified that PRKCA, as an upstream gene in the pathogenesis of atrial fibrosis, regulated downstream TGFβ1/Smad pathway, thereby promoting collagen secretion and transformation of atrial fibroblasts into myofibroblasts. In addition, some miRNAs, such as miR-21, miR-192 and miR-200a, have also been discovered to participate in tissue fibrosis by regulating TGFβ1/Smad signaling pathway.[35–37] Here, miR-647 has been proved as an upstream factor of this signaling pathway, affected the downstream TGFβ1/Smad pathway to participate in the process of AF related atrial fibrosis by targeting PRKCA gene.

Conclusion

This study elucidated the mechanism of miR-647 targeting PRKCA to regulate the activation of atrial fibroblasts through TGFβ1/Smad pathway. The results suggested that miR-647 regulation of PRKCA may be an important pathogenesis of AF, providing a new therapeutic target and experimental basis for the treatment of AF.

Declarations

Funding

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yang Yan and Dan Han. The first draft of the manuscript was written by Yang Yan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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Figures

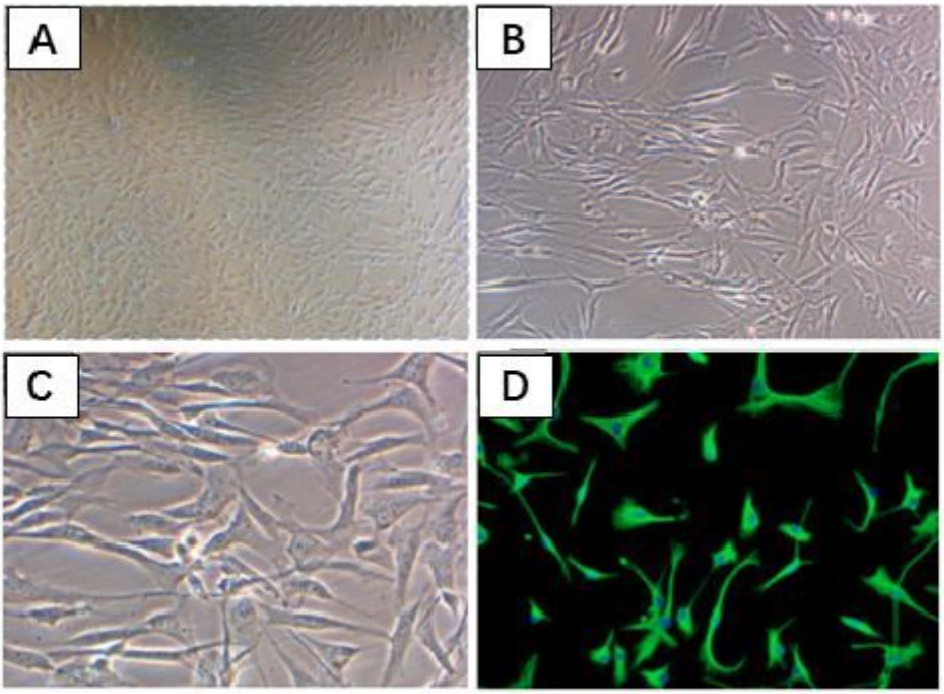

Figure 1

Identification of human atrial fibroblasts. (A) P0□10×; (B) P0□20×; (C) P0□40×; (D) P0□20×; green fluorescence is vimentin positive; blue fluorescence is DAPI.

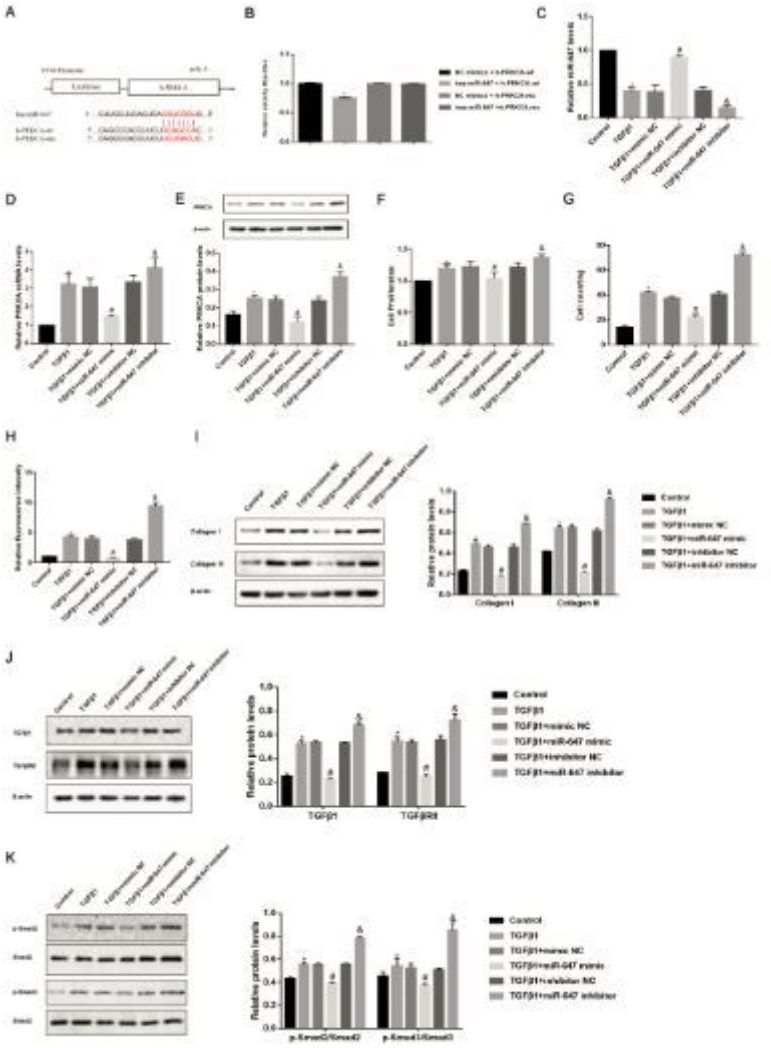

Figure 2

Effects of miR-647 expression on the proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation of TGFβ1-induced human atrial fibroblasts. (A) The putative binding sites between miR-647 and PRKCA. (B) Luciferase activity was measured in fibroblasts co-transfected with NC mimic or miR-647 mimic and PRKCA-wt or PRKCA-mu reporter at 48 h after transfection. MiR-647 targeted PRKCA directly, and inhibited the luciferase activity of PRKCA-wt. (C) The overexpression and knockdown efficiency of miR-647 was detected by qRT-PCR. (D, E) The mRNA and protein expression levels of PRKCA. MiR-647 overexpression obviously inhibited PRKCA expression. (F) Results of the CCK-8 assay showed that miR-647 overexpression inhibited the TGFβ1-induced cell proliferation, whereas miR-647 knockdown exerted the opposite effects. (G) Results of the transwell assay showed that miR-647 overexpression reduced the TGFβ1-induced cell migration, whereas miR-647 knockdown exerted the opposite effects. (H) Results of the immunofluorescence staining assay showed that miR-647 overexpression decreased the fluorescence intensity of α-SMA, whereas miR-647 knockdown exerted the opposite effects. (I) Results of the WB showed that miR-647 overexpression reduced collagen production,

whereas miR-647 knockdown exerted the opposite effects. (J, K) Results of the WB showed that miR-647 overexpression attenuated the activation of TGFβ1/Smad signaling pathway, whereas miR-647 knockdown exerted the opposite effects. (B)*P < 0.05 vs. NC mimic + PRKCA-wt; (C-I) *P < 0.05 vs. control, #P < 0.05 vs. TGFβ1 + mimic NC, &P < 0.05 vs. TGFβ1 + inhibitor NC. Data were presented as mean ± SD.

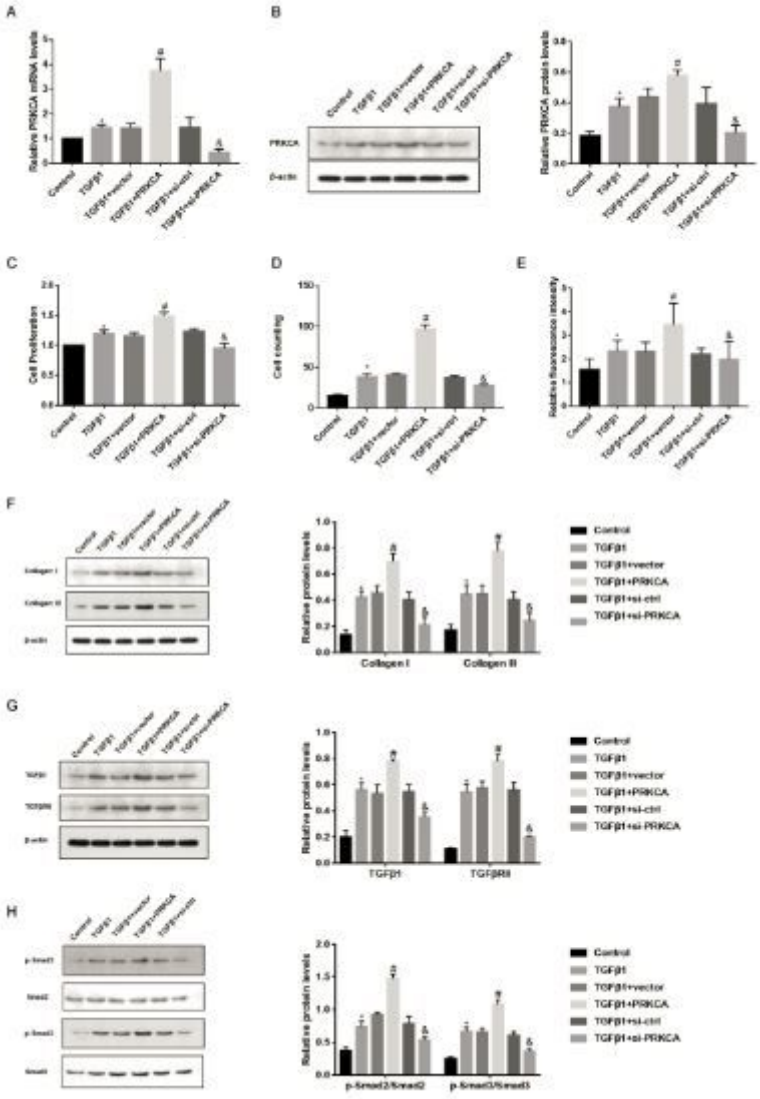

Figure 3

Effects of PRKCA expression on the proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation of TGFβ1-induced human atrial fibroblasts. (A) The overexpression and knockdown efficiency of PRKCA was detected by qRT-PCR. (B) The protein expression levels of PRKCA. (C) Results of the CCK-8 assay showed that PRKCA overexpression facilitated the TGFβ1-induced cell proliferation, whereas PRKCA knockdown exerted the opposite effects. (D) Results of the transwell assay showed that PRKCA overexpression promoted the TGFβ1-induced cell migration, whereas PRKCA knockdown exerted the opposite effects. (E) Results of the immunofluorescence staining assay showed that PRKCA overexpression increased the fluorescence intensity of α-SMA, whereas PRKCA knockdown

exerted the opposite effects. (F) Results of the WB showed that PRKCA overexpression increased collagen production, whereas PRKCA knockdown exerted the opposite effects. (G, H) Results of the WB showed that PRKCA overexpression promoted the activation of TGFβ1/Smad signaling pathway, whereas PRKCA knockdown exerted the opposite effects. *P < 0.05 vs. control, #P < 0.05 vs. TGFβ1 + vector, &P < 0.05 vs. TGFβ1 + si-ctrl. Data were presented as mean ± SD.

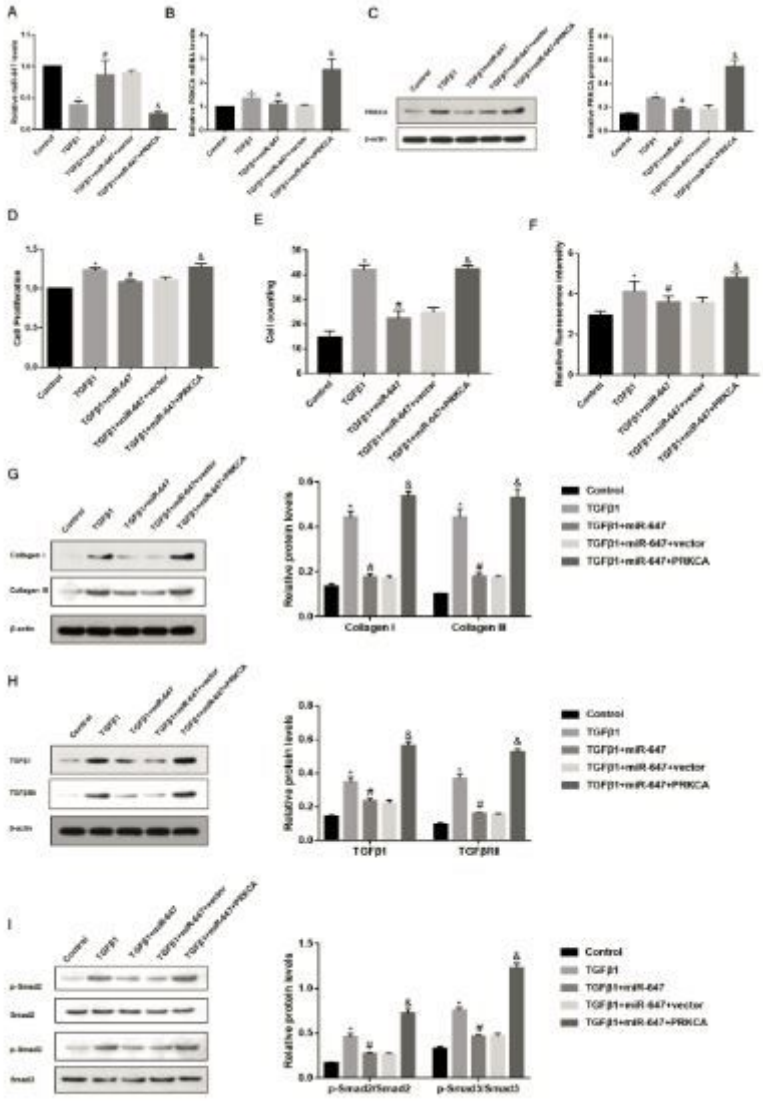

Figure 4

Effects of miR-647 expression on the proliferation, migration, transformation, collagen production, and TGFβ1/Smad signaling activation of TGFβ1-induced human atrial fibroblasts via PRKCA. (A) Relative miR-647 levels were detected by qRT-PCR. PRKCA overexpression significantly reduced the miR-647 expression level. (B, C) The mRNA and protein expression levels of PRKCA. Overexpression of miR-647 obviously reduced PRKCA expression. (D) Results of the CCK-8 assay showed that miR-647 overexpression suppressed the TGFβ1-induced cell proliferation, which was facilitated by PRKCA overexpression. (E) Results of the transwell assay showed that miR-647 overexpression inhibited the

TGFβ1-induced cell migration, which was promoted by PRKCA overexpression. (F) Results of the immunofluorescence staining assay showed that miR-647 overexpression decreased the fluorescence intensity of α-SMA, which was increased by PRKCA overexpression. (G) Results of the WB showed that miR-647 overexpression reduced collagen production, which was increased by PRKCA overexpression. (H, I) Results of the WB showed that miR-647 overexpression inhibited the activation of TGFβ1/Smad signaling pathway, which was facilitated by PRKCA overexpression. *P < 0.05 vs. control, #P < 0.05 vs. TGFβ1, &P < 0.05 vs. TGFβ1 + miR-647 + vector. Data were presented as mean ± SD.

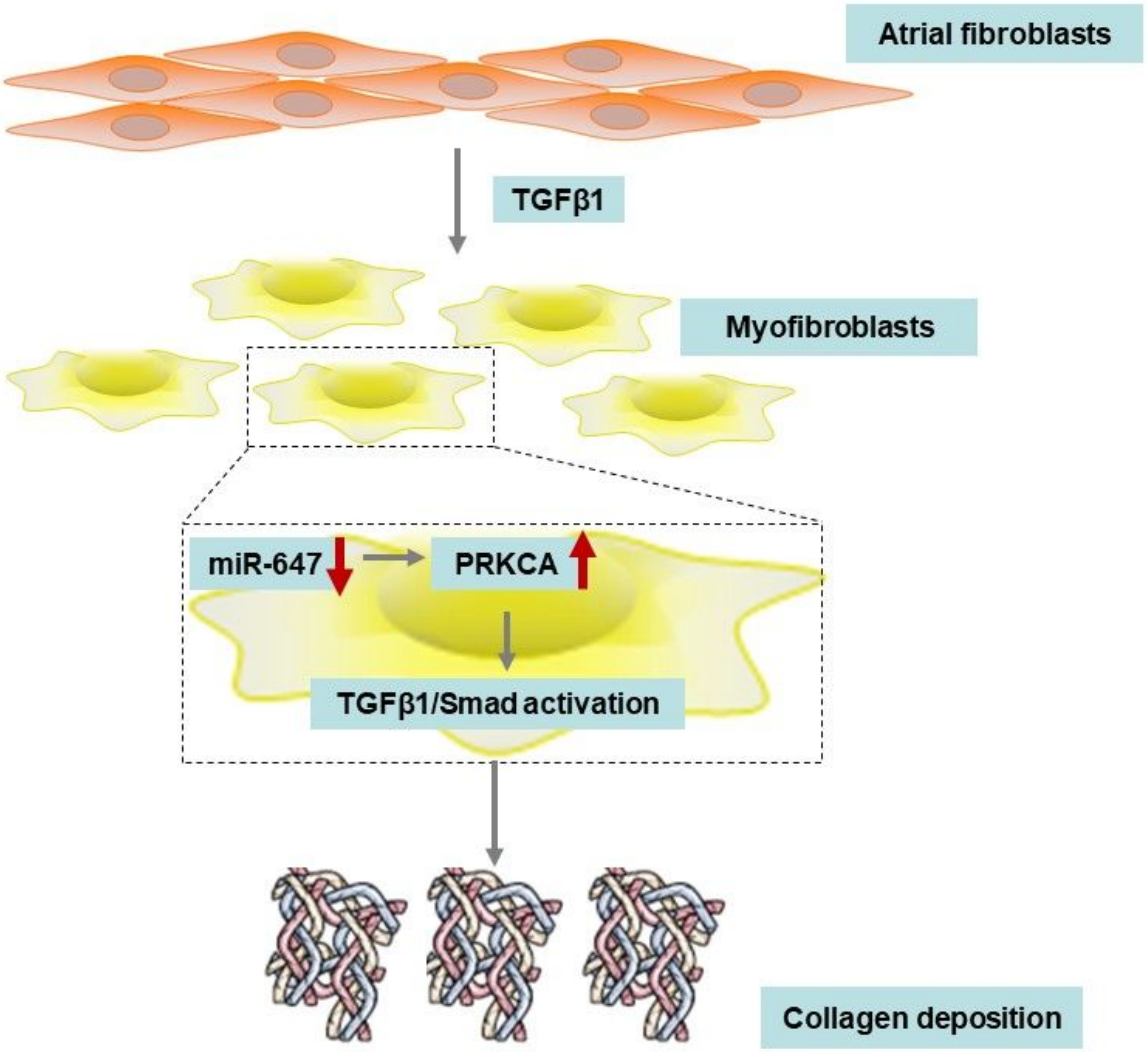

Figure 5

The mechanism diagram of our study. The human atrial fibroblasts were activated and transformed into myofibroblasts under TGFβ1 stimulation. The expression level of miR-647 decreased during the process. MiR-647 directly regulated PRKCA and therefore increased PRKCA expression. The activation of PRKCA increased the proliferation, migration, transformation, collagen production, and TGF-β1/Smad signaling activation of TGFβ1-induced human atrial fibroblasts. This study indicated that miR-647 regulated the activation of human atrial fibroblasts by targeting PRKCA.

Supplementary Files

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