LncRNA H19 Drives Proliferation of Cardiac Fibroblasts and Collagen Production via Suppression of the miR-29a-3p/miR-29b-3p-VEGFA/TGF-β Axis

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The aim of this study was to investigating whether lncRNA H19 promotes myocardial fibrosis by suppressing the miR-29a-3p/miR-29b-3p-VEGFA/TGF-β axis. Patients with atrial fibrillation (AF) and healthy volunteers were included in the study, and their biochemical parameters were collected. In addition, pcDNA3.1-H19, si-H19, and miR-29a/b-3p mimic/inhibitor were transfected into cardiac fibroblasts (CFs), and proliferation of CFs was detected by MTT assay. Expression of H19 and miR-29a/b-3p were detected using real-time quantitative polymerase chain reaction, and expression of α-smooth muscle actin (α-SMA), collagen I, collagen II, matrix metalloproteinase-2 (MMP-2), and elastin were measured by western blot analysis. The dual luciferase reporter gene assay was carried out to detect the sponging relationship between H19 and miR-29a/b-3p in CFs. Compared with healthy volunteers, the level of plasma H19 was significantly elevated in patients with AF, while miR-29a-3p and miR-29b-3p were markedly depressed ($P < 0.05$). Serum expression of lncRNA H19 was negatively correlated with the expression of miR-29a-3p and miR-29b-3p among patients with AF ($r_s = -0.337$, $r_s = -0.236$). Moreover, up-regulation of H19 expression and down-regulation of miR-29a/b-3p expression facilitated proliferation and synthesis of extracellular matrix (ECM)-related proteins. SB431542 and si-VEGFA are able to reverse the promotion of miR-29a/b-3p on proliferation of CFs and ECM-related protein synthesis. The findings of the present study suggest that H19 promoted CF proliferation and collagen synthesis by suppressing the miR-29a-3p/miR-29b-3p-VEGFA/TGF-β axis, and provide support for a potential new direction for the treatment of AF.

Keywords: atrial fibrillation, cardiac fibroblast proliferation, collagen synthesis, H19, miR-29a-3p, miR-29b-3p

INTRODUCTION

Atrial fibrillation (AF) is a common clinical arrhythmia that is characterized by decreased cardiac output, thromboembolism, stroke, and heart failure, all of which have a serious impact on human health. Global epidemiological data indicate there are currently around 33 million patients with AF, with an increase in prevalence of AF with increasing age (Rahman et al., 2014). Treatment options for AF are varied and

include medications and cardiac ablation, including atrioventricular node ablation and maze surgery. However, standard treatments currently available may not be wholly effective, resulting in either unresolved AF or recurrence (Bhatt and Fischer, 2015). Anatomical factors, ion channel and transport abnormalities and structural changes are associated with AF; however, the exact pathogenesis of AF, an important factor in guiding clinical treatment, has not been determined (Bhatt and Fischer, 2015).

Myocardial fibrosis (MF) is the most prominent type of structural remodeling in AF, and results in abnormal collagen fiber deposition in the extracellular matrix (ECM) of myocardial cells (Sheng et al., 2013). As MF processes, the proliferation of cardiac fibroblasts (CFs) increases, leading to an increases in the expression of type I collagen (Col I) and α-smooth muscle actin (α-SMA) (Tao et al., 2014c). It has been shown that long noncoding RNAs (lncRNAs) (Wang et al., 2015) and microRNAs (miRNAs) (Bauersachs, 2010) are involved in regulating the formation of MF, in addition to DNA methylation (Tao et al., 2014c) and histone deacetylation (Tao et al., 2014a). For example, lncRNA SENCR was found to be highly expressed in endothelial cells, vascular smooth muscle cells, and large artery tissues. Silencing SENCR might down-regulate expression of certain vascular smooth muscle contractile proteins (Uchida and Dimmeler, 2015). Furthermore, lncRNA Mhrt, has been found extensively in myocardial tissue and tends to hinder cardiac hypertrophy and heart failure under normal circumstances. Under conditions of pathology, however, its transcription is inhibited by the activation of the Brg1-Hdac-Parp chromatin inhibition complex (Han et al., 2014). Of note, the competing endogenous RNA (ceRNA) theory suggests that lncRNAs and miRNAs are mutually regulated by competitively binding to corresponding miRNA response elements (MREs), thereby affecting post-transcriptional regulation of miRNAs (Karreth et al., 2011; Salmena et al., 2011; Tay et al., 2011). In the etiology of myocardial hypertrophy, lncRNA CHRF was found to reduce miR-489 expression by sponging it (Wang et al., 2014). Furthermore, lncRNA H19 promoted the multiplication of CFs via repression of DUSP5/ERK1/2 signaling (Tao et al., 2016); however, the sponge miRNAs that could be a factor in AF etiology have rarely been studied.

With the use of ENCORI online software (http://starbase.sysu.edu.cn/), we discovered that the miR-29 family, including miR-29a, miR-29b, and miR-29c, was sponged by lncRNA H19. Members of the miR-29 family were shown to target mRNAs of various ECM proteins, such as type I/III collagen and fibrillin, and to control fibrosis-related genes, such as laminin and integrin. Maurer et al. (2010) discovered that miR-29a expression in fibroblasts of patients with systemic sclerosis was significantly down-regulated compared to that in healthy individuals. Another study demonstrated that miR-29b showed low expression in patients with AF or congestive heart failure (Dawson et al., 2013). Taken together, lncRNA H19 could be implicated in the development of AF by exerting inhibitory effects on the miR-29 family. However, no existing evidence to date has been able to corroborate the importance of the lncRNA H19/miR-29a/b axis in CF function and AF development.

The aim of this investigation, therefore, was to determine if lncRNA H19 is involved in AF development by sponging the miR-29 family, and to elucidate a potential new area for AF treatment.

MATERIALS AND METHODS

Clinical samples
Ninety-eight patients with AF from the cardiology department at Zhongda Hospital Southeast University, and 75 healthy volunteers were recruited into the study. Patients with AF were included if they met the following criteria: 1) irregular R-R intervals, 2) absence of distinct repeating P waves, and 3) irregular atrial activity, which was identified with an electrocardiogram (ECG) by a cardiologist. Patients with AF were grouped by pathology into paroxysmal AF (AF lasting < 7 days), persistent AF (AF that persists for > 7 days), and permanent AF (AF that persists for > 12 months), according to the "2014 AHA/ACC/HRS Guidelines for the Management of Patients with Atrial Fibrillation" (January et al., 2014).

Patients with AF were excluded from the study if they had the following co-morbid disorders: malignant tumors, infectious diseases, connective tissue diseases, autoimmune diseases, Parkinson's syndrome, Alzheimer's disease, primary biliary cirrhosis, chronic bladder pain syndrome or severe renal diseases. In addition, patients with AF that occurred after surgery and during pregnancy, or was complicated by acute myocardial infarction, hyperthyroidism and hypertrophic cardiomyopathy, were also excluded. All research subjects volunteered for this study, and all participants signed informed consent forms prior to enrolment. The protocol for this study was approved by Zhongda Hospital Southeast University and the Ethics Committee of Zhongda Hospital Southeast University (No. 2019ZDKYSB057).

Collection of blood samples from participants
Upon admission, elbow venous blood was taken from each participant, who was instructed to fast for more than 12 h prior to the blood draw. An automatic biochemistry analyzer (Roche, Switzerland) was used to measure serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), creatinine (Cr), and high-sensitivity C-reactive protein (hs-CRP).

Measurement of left atrial diameter and left ventricular ejection fraction (%)
The left atrial diameter (LAD) and left ventricular ejection fraction (LVEF) were obtained by echocardiography performed with the Cardiovascular Ultrasound System (VIVID T8; GE Healthcare, USA).

Cell culture
Rat CFs (purchased from Shanghai iCell Bioscience, China), were incubated in 15% fetal bovine serum-containing DMEM, which included penicillin-streptomycin, and were placed in an atmosphere of 5% CO_2 at 37°C. After the cells grew to 80% confluence, they were digested by 0.25% trypsin (Beyotime Institute of Biotechnology, China). The cells

that were passaged for three generations were prepared for subsequent experiments.

Cell treatment and transfection

CFs of rats were transfected, respectively, by pcDNA3.1-lncRNA H19, si-lncRNA H19 (siRNA-1, F: 5'-TGACGGCGAGG-ACAGAGGAG-3'; R: 5'-CCCAGAGGGCAGCCATAGTG-3'; siRNA-2, F: 5'-CCCACAACAUGAAAGAAACTT-3'; R: 5' AUUUCUUUCAUGUUGUGG-Grl 3'; siRNA-3, F: 5'-GCUA-GAGGAACCAGACCUUTT-3'; R: 5'-AAGGUCUGGUUC-CUCUAGCTT-3'), miR-29a-3p mimic (F: 5'-UAGCACCAU-CUGAAAUCGGUUA-3'; R: 5′-ACCGAUUUCAGAUGGU-GCUAUU-3′), miR-29a-3p inhibitor (F: 5′-UAACCGAUUU-CAGAUGGUGCUA-3′; R: 5′-CAGUACUUUUGUGUAGUA-CAA-3′), miR-29b-3p mimic (F: 5'-UAGCACCAUUUGAAAU-CAGUGUU-3'; R: 5'-AACACUGAUUUCAAAUGGUGCUA-3'), and miR-29b-3p inhibitor (5'-AACACUGAUUUCAAAUGGU-GCUA-3'), according to the instructions of the Lipofectamine 2000 transfection reagent kit (Invitrogen, USA).

Cell proliferation assay

CFs seeded in 96-well plates were blended with a 5 mg/ml MTT solution (Beijing Solarbio Science & Technology, China), after which they were cultivated, respectively, for 0 h, 24 h, 48 h, and 72 h. After continuous incubation of CFs for 4 h, the cell supernatant was removed, and CFs from each well were mixed with 150 μl dimethyl sulfoxide (Amresco, USA) and shaken for 10 min. The optical density of the CFs was measured at 490 nm with a microplate reader (Bio-Tek, USA).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA in CFs and blood samples was extracted by the addition of Trizol reagent (Beijing Tiangen Biochemical Technology, China). After synthesizing cDNAs according to the instructions of the reverse transcription kit (TaKaRa, Japan), the cDNAs were then amplified as instructed by a real-time fluorescent quantitative PCR kit (TaKaRa). Accordingly, $2^{-\triangle\triangle CT}$ method was employed to standardize gene expressions, with GAPDH and U6 as the internal reference.

Western blot analysis

The CFs were washed twice with pre-chilled phosphate-buffered saline (PBS), and subsequently lysed on ice in order to dissociate the proteins from the CFs. After determining the protein concentration using the BCA method, 40 μg of protein sample was removed in order to carry out electrophoresis, followed by transfer onto a PVDF membrane (Millipore, France). After blockage by 5% skimmed milk powder for 1 h, primary antibodies (rabbit anti-rat; Abcam, USA) against Bax (1:2,000, Cat. No. ab32503), Bcl-2 (1:1,000, Cat. No. ab32124), caspase-3 (1:5,000, Cat. No. ab32351), α-SMA (1:10,000, Cat. No. ab124964), collagen I (1:3,000, Cat. No. ab34710), collagen II (1:5,000, Cat. No. ab188570), MMP-2 (1:1,000, Cat. No. ab92536), elastin (1:1,000, Cat. No. ab213720), and GAPDH (1:2,500, Cat. No. ab9485) were added and the samples were incubated at 4°C overnight. After washing the membrane with PBST, a secondary antibody Goat Anti-Rabbit IgG H&L (HRP) (1:3,000, Cat. No. ab205718; Abcam) was added to incubate the resultants, and GAPDH was used as the control.

Dual luciferase reporter gene assay

CFs in the logarithmic growth phase was seeded in 24-well cell plates at a density of 5 × 10^4/well. With the assistance of a dual luciferase reporter gene detection kit (lot No. E1913; Promega, USA), the pmirGLO-H19-Wt (Promega) and pmir-GLO-H19-Mut were co-transfected, respectively, with miR-29a-3p/miR-29b-3p mimic, miR-29a-3p/miR-29b-3p inhibitor or miR-negative control (miR-NC) into CFs for 48 h. Relative luciferase activity, i.e., the ratio of reninase activity/luciferase

Table 1. Comparison of baseline features between atrial fibrillation patients and healthy people

Clinical characteristic	Atrial fibrillation group	Control group	χ^2/t-test	P value
Sex			0.046	0.963
Female	48	37		
Male	50	38		
Age (y)	61.64 ± 7.05	60.12 ± 6.86	1.422	0.157
Coronary heart disease	16 (16.3)	20 (26.7)	2.757	0.097
Diabetes mellitus	8 (8.2)	13 (17.3)	3.350	0.067
Hypertension	27 (27.6)	25 (33.3)	0.676	0.411
Smoking	20 (20.4)	14 (18.7)	0.082	0.775
LAD (mm)	44.89 ± 5.42	32.18 ± 4.26	16.73	<0.001
LVEF (%)	52.19 ± 6.13	63.75 ± 5.96	12.44	<0.001
TC (mmol/L)	4.06 ± 0.90	3.83 ± 1.21	1.434	0.153
TG (mmol/L)	1.42 ± 0.42	1.30 ± 0.53	1.661	0.098
LDL-C (mmol/L)	2.93 ± 0.49	2.27 ± 0.38	9.651	<0.001
HDL-C (mmol/L)	1.18 ± 0.41	1.10 ± 0.22	1.529	0.128
hs-CRP (mg/L)	5.07 ± 3.02	1.16 ± 0.47	11.100	<0.001
Serum creatinine (μmol/L)	68.58 ± 14.08	63.19 ± 12.61	2.609	0.010

Values are presented as number only, mean ± SD, or number (%).
LAD, left atrial diameter; LVEF, left ventricular ejection fraction; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein.

activity, of CFs in each group was determined.

Statistical analyses

The data were statistically analyzed with SPSS 17.0 software (SPSS, USA). Numerical variables are expressed as mean ± SD, and were compared with the use of the Pearson chi-square test or one-way ANOVA. Statistical significance was defined as $P < 0.05$.

RESULTS

Clinical traits of AF patients

There were few, if any, significant differences between patients with AF and healthy individuals, with regards to gender, age, history of coronary heart disease, history of diabetes, history of hypertension, and smoking history ($P > 0.05$). However, LAD, LDL-C, and hs-CRP were significantly increased, and LVEF was significantly decreased in patients with AF, compared to that in healthy individuals ($P < 0.05$) (Table 1). Furthermore, LAD, LVEF, LDL-C, and hs-CRP increased with AF duration ($P < 0.05$) (Table 2).

In order to characterize the potential role of H19, miR-29a-3p, and miR-29b-3p in AF progression, their expression was measured by RT-qPCR. Serum levels of lncRNA H19 in AF patients increased to 1.60 times that of the control group, but miR-29a-3p and miR-29b-3p expression in the serum of AF patients decreased to only 39.95%-52.17% of that in the control group. LncRNA H19 expression increased, along with a decreased expression of miR-29a-3p and miR-29b-3p, as AF was continued ($P < 0.05$) (Fig. 1A). Serum expression of lncRNA H19 was negatively correlated with expressions of miR-29a-3p and miR-29b-3p among AF patients ($r_s = -0.337$,

$r_s = -0.236$) (Fig. 1B). These data showed that the lncRNA H19 expression was significantly increased, but the miR-29a-3p and miR-29b-3p expression was clearly decreased in the AF patients. Within the AF population, lncRNA H19 expression was positively correlated with LAD, LDL-C, and hs-CRP ($r_s = 0.295$, $r_s = 0.284$, $r_s = 0.348$), and negatively correlated with LVEF ($r_s = -0.227$) (Fig. 2A). Conversely, expression of either miR-29a-3p or miR-29b-3p was negatively correlated with LAD, LDL-C, and hs-CRP ($r_s = -0.319$, $r_s = -0.299$, $r_s = -0.342$; $r_s = -0.485$, $r_s = -0.424$, $r_s = -0.375$), and positively correlated with LVEF ($r_s = 0.296$; $r_s = 0.497$) (Figs. 2B and 2C). However, H19, miR-29a-3p, and miR-29b-3p had no effect on TC, TG, HDL-C, and serum creatinine.

LncRNA H19 sponged miR-29a-3p/miR-29b-3p to regulate CF activity and collagen production

To explore the functional roles of lncRNA H19, miR-29a-3p, and miR-29b-3p in CF cell proliferation, apoptosis and collagen production, CF cells were transfected with either a control vector, pcDNA3.1-H19, si-H19, a mimic or an inhibitor. H19 expression in CFs of the pcDNA3.1-H19 group increased to 6.05 times that of the control group, while siRNA-1, siRNA-2, and siRNA-3 inhibited H19 expression in CFs by 22%-53% of that in the control group ($P < 0.05$) (Fig. 3A). Among the siRNAs, siRNA-1 demonstrated the strongest degree of inhibition and was used for subsequent experiments. Furthermore, miR-29a-3p expression was promoted by an miR-29a-3p mimic, and suppressed under the influence of an miR-29a-3p inhibitor ($P < 0.05$) (Fig. 3B). Analogously, miR-29b-3p expression was up-regulated in the miR-29b-3p mimic group, and down-regulated in the miR-29b-3p inhibitor group, when compared with the NC group ($P < 0.05$) (Fig.

Table 2. Comparison of baseline features among patients with paroxysmal atrial fibrillation, persistent atrial fibrillation and permanent atrial fibrillation

Clinical characteristic	Paroxysmal atrial fibrillation group	Persistent atrial fibrillation group	Permanent atrial fibrillation	P value
Sex				
Female	21	13	14	0.037[a]
Male	15	20	15	
Age (y)	59.73 ± 6.43	61.92 ± 6.97	63.69 ± 7.48	0.075
Coronary heart disease	5	6	5	0.879[a]
Diabetes mellitus	3	3	2	0.951[a]
Hypertension	9	10	8	0.886[a]
Smoking	6	8	6	0.737[a]
LAD (mm)	41.35 ± 4.27	44.21 ± 3.67	50.05 ± 4.38	<0.001[b]
LVEF (%)	55.46 ± 6.02	52.24 ± 5.21	48.07 ± 4.73	<0.001[b]
TC (mmol/L)	4.15 ± 0.74	4.08 ± 0.91	3.92 ± 1.07	0.588[b]
TG (mmol/L)	1.38 ± 0.40	1.51 ± 0.48	1.37 ± 0.35	0.321[b]
LDL-C (mmol/L)	2.68 ± 0.37	2.94 ± 0.42	3.22 ± 0.55	<0.001[b]
HDL-C (mmol/L)	1.19 ± 0.51	1.22 ± 0.34	1.12 ± 0.36	0.630[b]
hs-CRP (mg/L)	3.46 ± 1.62	5.06 ± 2.27	7.10 ± 3.84	<0.001[b]
Serum creatinine (μmol/L)	64.71 ± 13.71	69.64 ± 13.90	72.19 ± 14.04	0.089[b]

Values are presented as number only, mean ± SD, or number (%).
The results are statistically significant when the $P < 0.05$.
LAD, left atrial diameter; LVEF, left ventricular ejection fraction; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein.
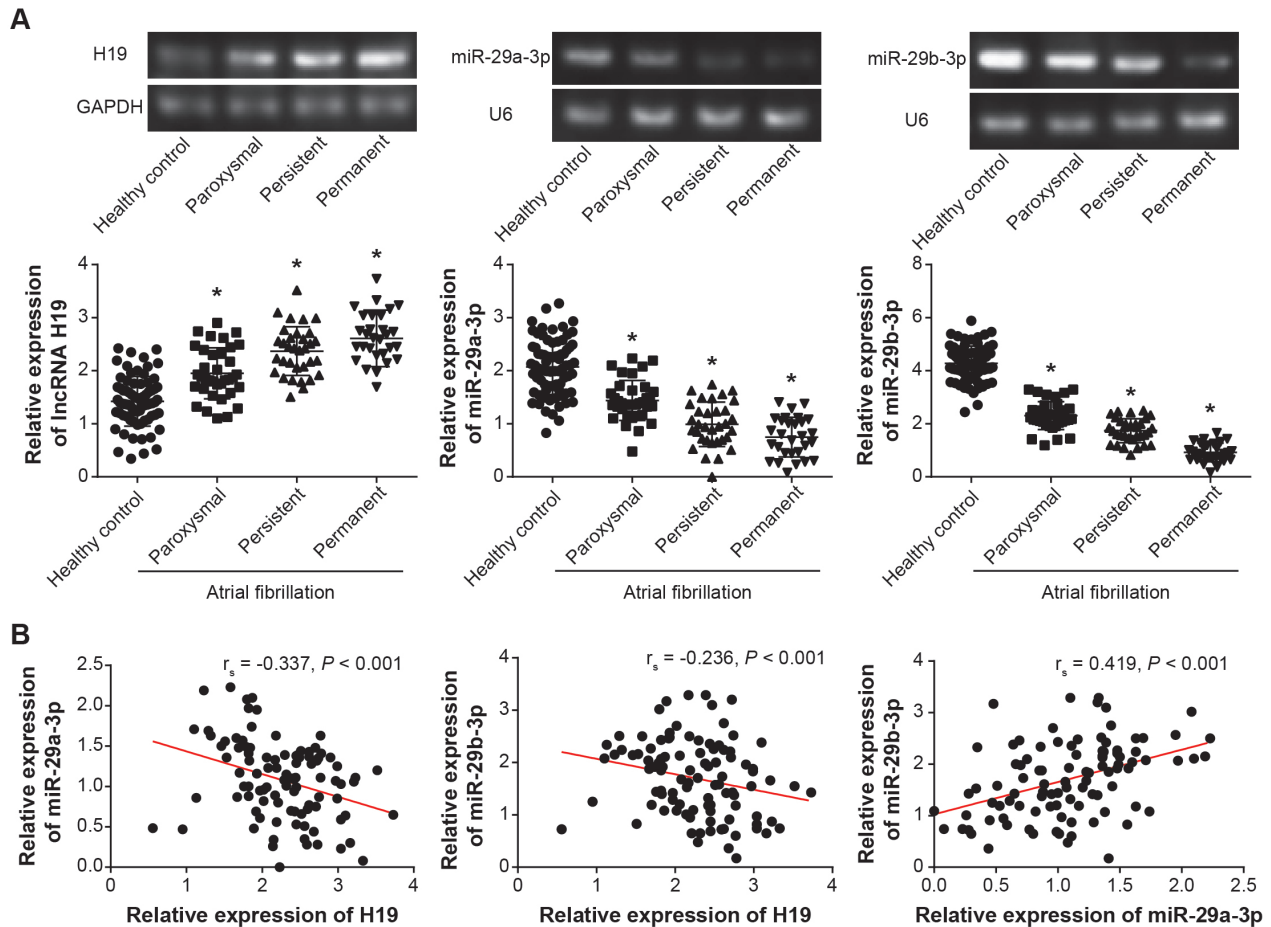[a]Pearson chi-square test. [b]One-way ANOVA.

Fig. 1. Clinical significance of lncRNA H19, miR-29a-3p, and miR-29b-3p in atrial fibrillation (AF). (A) Serum levels of lncRNA H19, miR-29a-3p, and miR-29b-3p were compared between AF patients and healthy volunteers. *$P < 0.05$ when compared with healthy control. (B) Serum level of lncRNA H19 was negatively correlated with that of miR-29a-3p and miR-29b-3p among AF patients.

3C).

Compared with the NC group, the viability and proliferation of CFs in the pcDNA3.1-H19 group and miR-29a-3p/miR-29b-3p inhibitor group was enhanced, whereas si-H19 and the miR-29a-3p/miR-29b-3p mimic reduced the viability and proliferative capability of CFs ($P < 0.05$) (Figs. 3D-3G). The results show that cell proliferation rate and colony formation ability in the pcDNA3.1-H19, miR-29a-3p/miR-29b-3p inhibitor group were significantly higher compared to that in the control group. On the contrary, apoptosis of CFs in the si-H19 group or in the miR-29a-3p/miR-29b-3p mimic group was significantly facilitated in comparison to the NC group; however, the apoptotic tendency of CFs in the pcD-NA3.1-H19 group and miR-29a-3p/miR-29b-3p inhibitor was reversed, relative to that of the NC group ($P < 0.05$) (Figs. 4A and 4B). Similarly, the expression profile of apoptosis-related proteins was also changed. More specifically, pcDNA3.1-H19 and miR-29a-3p/miR-29b-3p inhibitors led to under-expression of Bax and cleaved caspase-3/total caspase-3, as well as over-expression of Bcl-2 ($P < 0.05$) (Figs. 4C and 4D). In contrast, transfection of si-H19 and miR-29a-3p/miR-29b-3p mimics increased expression of the Bax/caspase-3 ratio and

decreased Bcl-2 expression ($P < 0.05$). Moreover, expression levels of α-SMA, collagen I, collagen II, MMP-2, and elastin in the pcDNA3.1-H19 group and miR-29a-3p/miR-29b-3p inhibitor group were increased to 1.86-3.03 times of NC group, while expressions of α-SMA, collagen I, collagen II, MMP-2, and elastin were significantly suppressed compared to the NC group ($P < 0.05$) (Fig. 5). The current results indicate that H19 overexpression and miR-29a-3p/miR-29b-3p under-expression significantly promote cell proliferation and collagen production, while inhibiting apoptosis in CF cells.

Sponging relationship between lncRNA H19 and miR-29a-3p/miR-29b-3p

Having determined the potential competitive mechanism between H19 and miR-29a-3p/miR-29b-3p, the present study attempted to identify critical direct targets underlying the mechanistic contribution of H19, miR-29a-3p, and miR-29b-3p to the progression of AF. The predicted binding site of miR-29a-3p/miR-29b-3p at the 3'-UTR of H19 site was determined using the starBase online database. The potential binding sequences are shown in Figs. 6A and 6C. A dual-luciferase reporter assay was conducted to validate direct
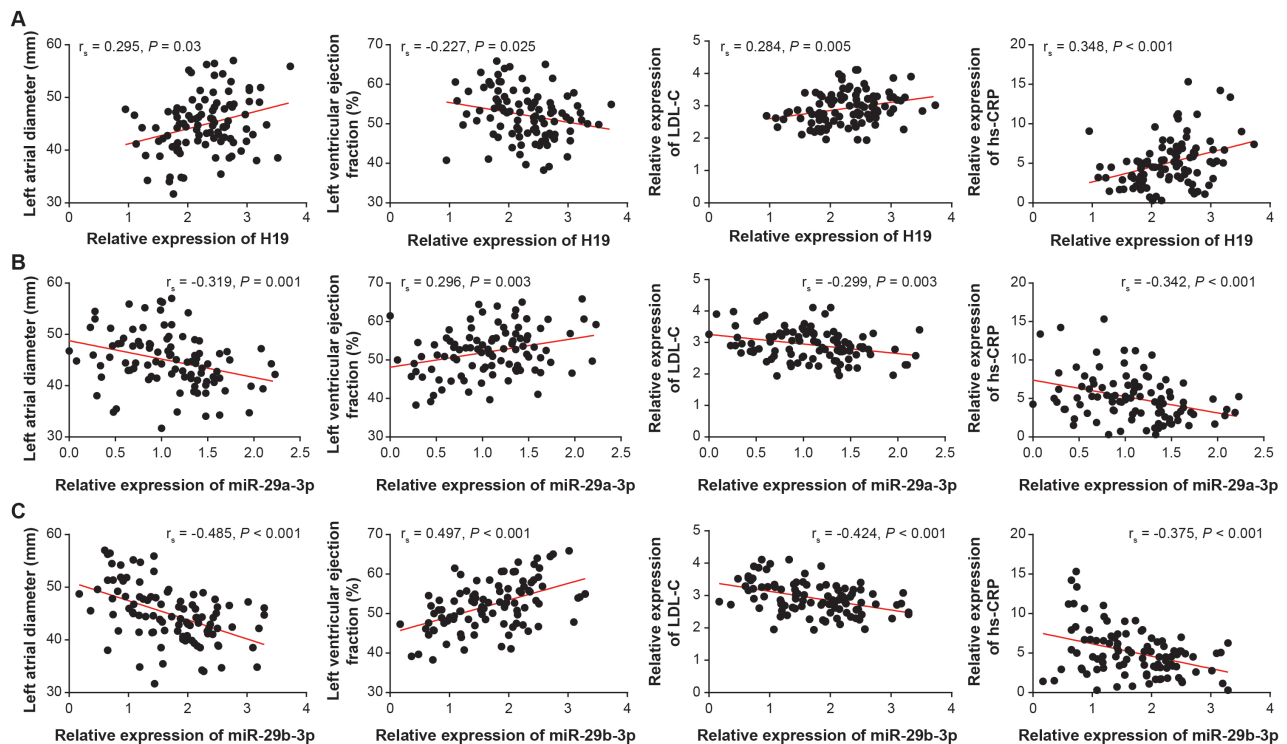
Fig. 2. Correlations between expression of RNAs and clinical traits of AF patients. Serum levels of lncRNA H19 (A), miR-29a-3p (B), and miR-29b-3p (C) were correlated with clinical traits of AF patients.

binding of the 3'-UTR of H19 mRNA with miR-29a-3p/miR-29b-3p. The luciferase activity of CFs in the pmiRGLO-H19-Wt+miR-29a-3p/miR-29b-3p mimic group was markedly restrained, when compared with the combined transfection of the pmiRGLO-H19-Mut-miR-29a-3p/miR-29b-3p mimic group and the pmiRGLO-H19-Wt-miR-NC group ($P < 0.05$) (Figs. 6A and 6C). Furthermore, miR-29a-3p/miR-29b-3p expression was significantly inhibited after transfection of pcDNA3.1-H19 ($P < 0.05$), and increased under the influence of si-H19 ($P < 0.05$) (Figs. 6B and 6D). In summary, the results from this study suggest that H19 regulate AF progression by binding to miR-29a-3p/miR-29b-3p.

MiR-29a-3p mediated the effect of lncRNA H19 on CF activity and collagen production by targeting VEGFA and regulating TGF-β

To further verify that miR-29a-3p regulates the activities of CF cells by targeting VEGFA and TGF-β, miR-29a-3p inhibitor alone or miR-29a-3p inhibitor combined with si-VEGFA or SB431542 was transfected into CF cells. The viability and multiplicative potential of CFs was enhanced, and CF apoptosis was slowed down in the miR-29a-3p inhibitor group comparison to the miR-29a-3p inhibitor+si-VEGFA group ($P < 0.05$) (Figs. 7A-7C). Moreover, CFs in the miR-29a-3p inhibitor+si-VEGFA group produced higher expressions of Bax and cleaved caspase-3/total caspase-3 and lower Bcl-2 expression than those in the miR-29a-3p inhibitor group ($P < 0.05$) (Fig. 7D). Furthermore, expressions of α-SMA, collagen I, collagen II, MMP-2 and elastin were lowered under the combined ef-

fect of miR-29a-3p inhibitor and si-VEGFA, as relative to miR-29a-3p inhibitor group ($P < 0.05$) (Fig. 7E).

In addition, the viability and proliferation of CFs was markedly prohibited in the miR-29a-3p inhibitor+SB431542 group when compared to the with miR-29a-3p inhibitor group ($P < 0.05$) (Supplementary Figs. S1A and S1B), whereas CF apoptosis was promoted under the co-treatment of miR-29a-3p inhibitor and SB431542 compared to the miR-29a-3p inhibitor group ($P < 0.05$) (Supplementary Fig. S1C). CFs of the miR-29a-3p inhibitor+SB431542 group were associated with decreases in Bcl-2 expression, along with increases in Bax expression and cleaved caspase-3/total caspase-3 ratio, relative to the miR-29a-3p inhibitor group ($P < 0.05$) (Supplementary Fig. S1D). Expressions of α-SMA, collagen I, collagen II, MMP-2 and elastin decreased in the miR-29a-3p inhibitor+SB431542 group compared to miR-29a-3p inhibitor group ($P < 0.05$) (Supplementary Fig. S1E).

The aforementioned results demonstrate that miR-29a-3p inhibitor promoted proliferation and collagen production, and inhibited apoptosis. This inhibition of apoptosis could be abolished with si-VEGFA/SB43152, suggesting that miR-29a-3p exerts its effects on cell proliferation, collagen production and cell apoptosis through VEGFA/TGF-β.

MiR-29b-3p participated in the effect of lncRNA H19 on CF activity and collagen production by targeting TGF-β and modifying VEGFA

To demosntrate the effect of miR-29b-3p on CF activities and collagen production through VEGFA and TGF-β, miR-29b-3p
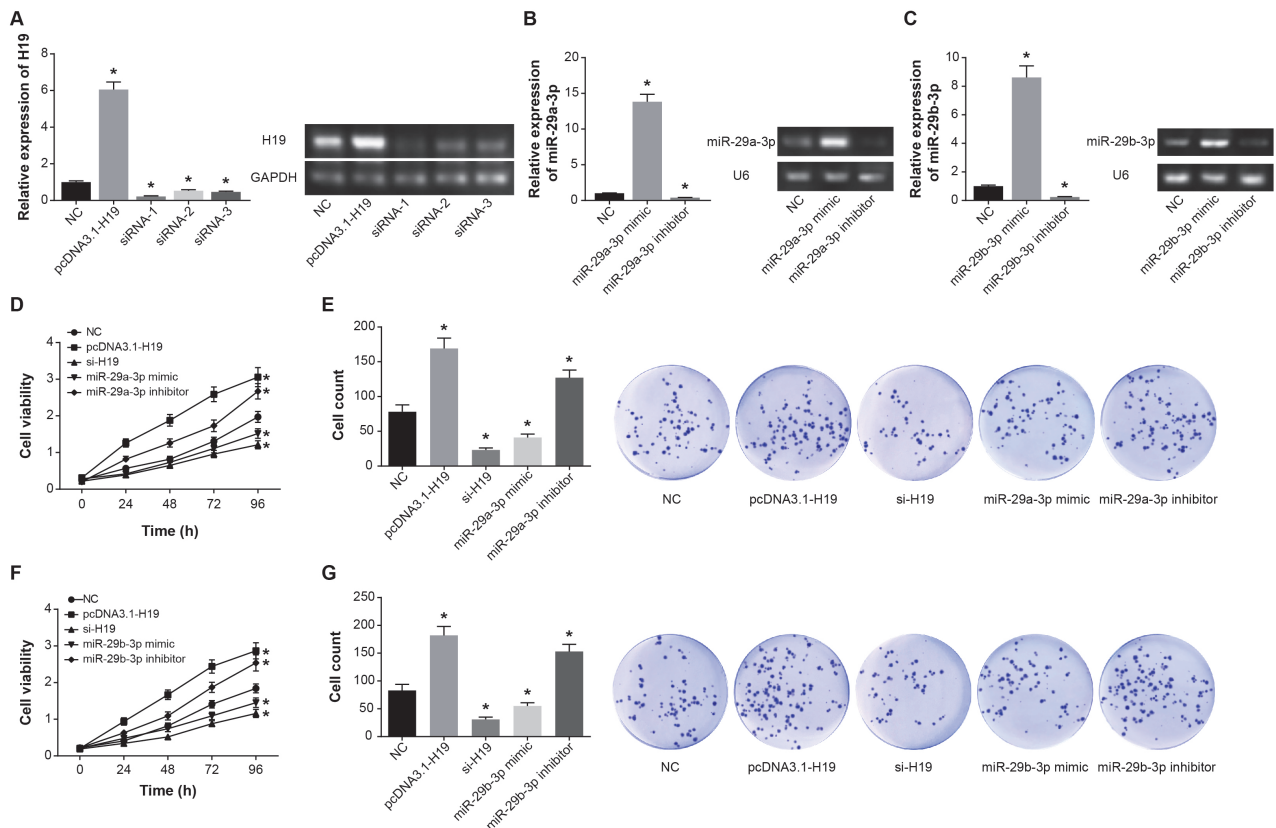
Fig. 3. Cell activities were impacted by lncRNA H19, miR-29a-3p, and miR-29b-3p in CFs. (A) LncRNA H19 expression was determined after transfection of pcDNA3.1-H19, siRNA-H19-1, siRNA-H19-2, and siRNA-H19-3. ∗$P < 0.05$ when compared with negative control (NC). (B) MiR-29a-3p expression in CFs was detected after transfection of miR-29a-3p mimic and miR-29a-3p inhibitor. ∗$P < 0.05$ when compared with NC. (C) MiR-29a-3p expression was detected after transfection of miR-29b-3p mimic and miR-29b-3p inhibitor into CFs. ∗$P < 0.05$ when compared with NC. (D-G) Viability and proliferation of CFs was evaluated among NC, pcDNA3.1-H19, si-H19, miR-29a-3p mimic and miR-29a-3p inhibitor group or NC, pcDNA3.1-H19, si-H19, miR-29b-3p mimic, and miR-29b-3p inhibitor group. ∗$P < 0.05$ when compared with NC.

inhibitor alone or combined with si-VEGFA or SB431542 was transfected into CF cells. CFs of miR-29b-3p inhibitor+TGF-β1 blocker (SB431542) group were associated with weaker viability and proliferative ability than CFs of the miR-29b-3p inhibitor group ($P < 0.05$) (Figs. 8A and 8B). Combined treatment of miR-29b-3p inhibitor and SB431542 contributed to a significant increase in the rate of apoptosis, which was 1.63 times of that in the miR-29b-3p inhibitor group ($P < 0.05$) (Fig. 8C). Joint treatment of miR-29b-3p inhibitor and SB431542 up-regulated expression of Bax and cleaved caspase-3/total caspase-3 and down-regulated Bcl-2 expression, when compared with the miR-29b-3p inhibitor group ($P < 0.05$) (Fig. 8D). Moreover, SB431542 treatment (i.e., miR-29b-3p inhibitor+SB431542 group) reversed the contribution of miR-29b-3p inhibitor to collagen production, resulting in lower expressions of α-SMA, collagen I, collagen II, MMP-2 and elastin than miR-29b-3p inhibitor treatment alone ($P < 0.05$) (Fig. 8E).

In addition, CFs of miR-29b-3p inhibitor+si-VEGFA group revealed weaker viability and proliferation, as well as a higher apoptotic rate, than CFs of the miR-29b-3p inhibitor group

($P < 0.05$) (Supplementary Figs. S2A-S2C). Bcl-2 expression was down-regulated; however, Bax expression and cleaved caspase-3/total caspase-3 ratio was up-regulated in CFs of the miR-29b-3p inhibitor+si-VEGFA group, when compared with the miR-29b-3p inhibitor group ($P < 0.05$) (Supplementary Fig. S2D). Double treatment of miR-29b-3p inhibitor and si-VEGFA led to lower expressions of α-SMA, collagen I, collagen II, MMP-2, and elastin than miR-29b-3p inhibitor transfection alone ($P < 0.05$) (Supplementary Fig. S2E).

The above results showed that miR-29b-3p inhibitor promoted cell proliferation and collagen production, and inhibited apoptosis. These effects were reversed by si-VEGFA/SB43152. It was also shown that miR-29b-3p modified cell proliferation, collagen production and cell apoptosis through VEGFA/TGF-β.

DISCUSSION

AF is one of the most common subtypes of arrhythmia, occurring in 0.4% of the general population, with an incidence reaching high of 8% in elderly populations aged 80 years or
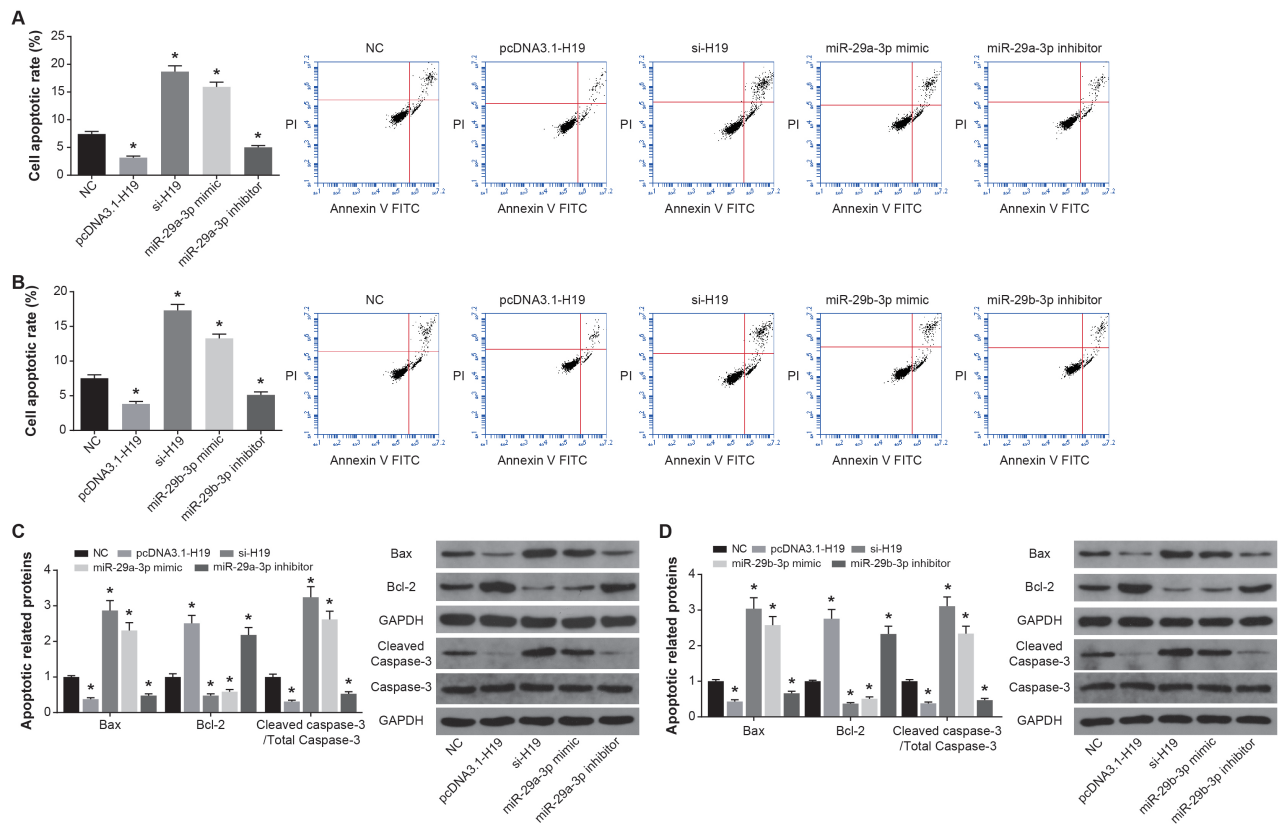
Fig. 4. Cell apoptosis were regulated by lncRNA H19, miR-29a-3p, and miR-29b-3p in CFs. (A and B) apoptosis were evaluated separately among negative control (NC), pcDNA3.1-H19, si-H19, miR-29a-3p mimic, and miR-29a-3p inhibitor group or NC, pcDNA3.1-H19, si-H19, miR-29b-3p mimic, and miR-29b-3p inhibitor group. *P < 0.05 when compared with NC. PI, propidium iodide; FITC, fluorescein isothiocyanate. (C and D) apoptins were determined between NC, pcDNA3.1-H19, si-H19, miR-29a-3p mimic, and miR-29a-3p inhibitor group and NC, pcDNA3.1-H19, si-H19, miR-29b-3p mimic, and miR-29b-3p inhibitor group. *P < 0.05 when compared with NC.
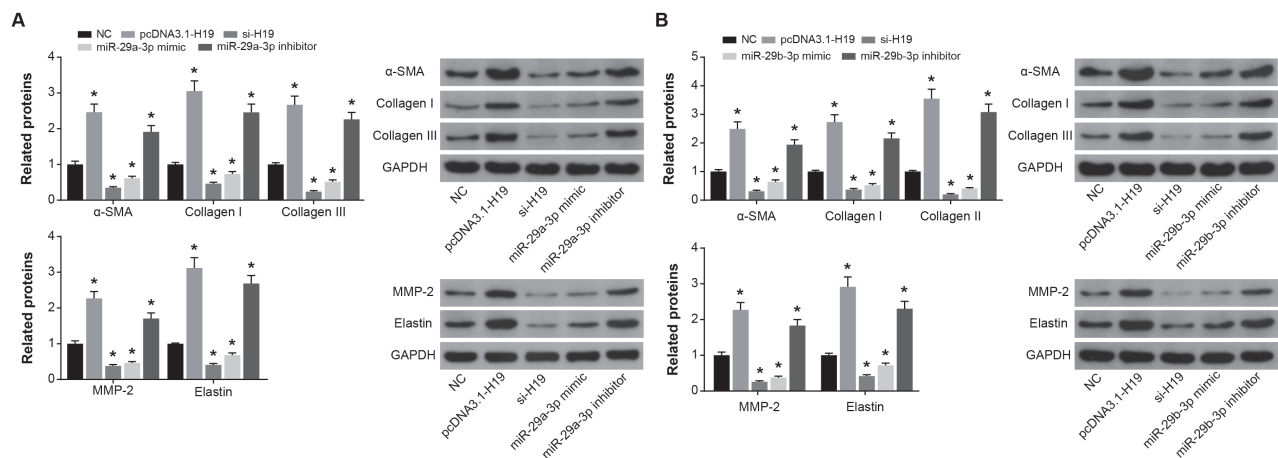

Fig. 5. ECM-related proteins of CFs were affected by lncRNA H19, miR-29a-3p, and miR-29b-3p. (A) ECM-related proteins were evaluated separately among negative control (NC), pcDNA3.1-H19, si-H19, miR-29a-3p mimic, and miR-29a-3p inhibitor group. *P < 0.05 when compared with NC. (B) ECM-related proteins were detected among negative NC, pcDNA3.1-H19, si-H19, miR-29b-3p mimic, and miR-29b-3p inhibitor group. *P < 0.05 when compared with NC.
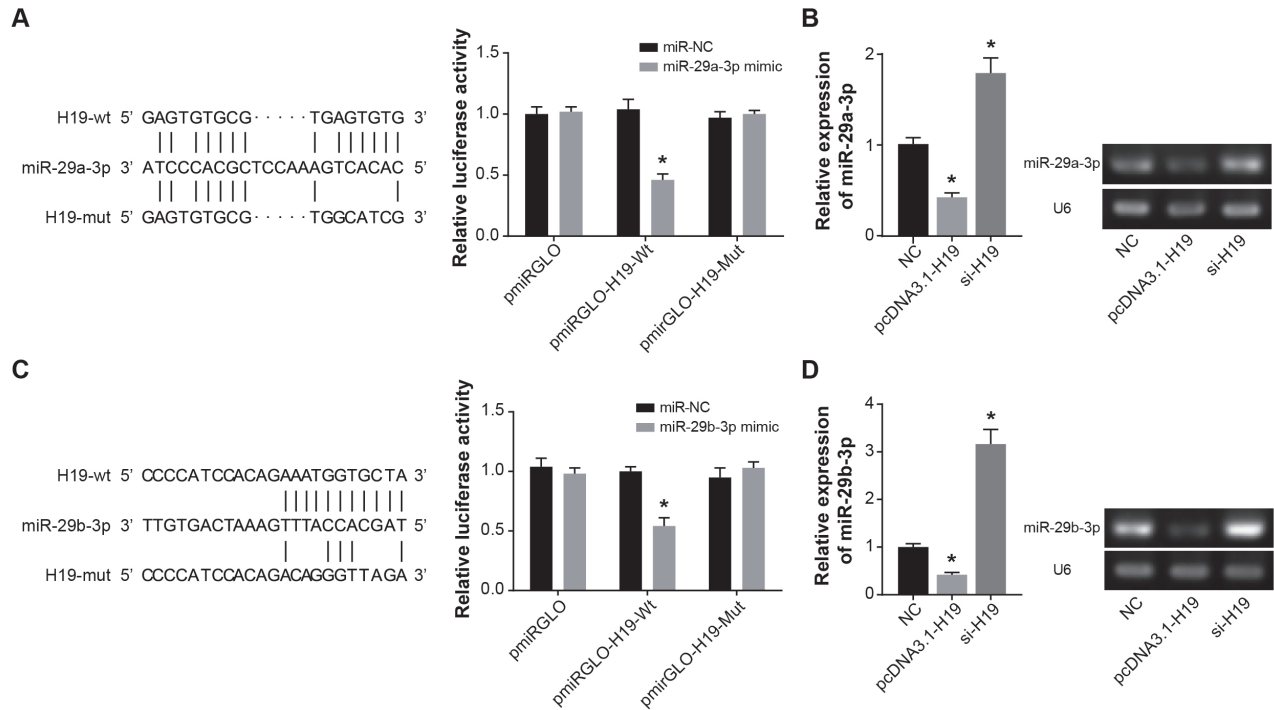
Fig. 6. Sponging relationships between H19 and miR-29a-3p/miR-29b-3p in CFs. (A) LncRNA H19 targeted miR-29a-3p, and luciferase activity of CFs was compared between pmiRGLO-H19-Wt+miR-29a-3p mimic group and pmiRGLO-H19-Wt+miR-NC group. NC, negative control. *$P < 0.05$ when compared with pmiRGLO-H19-Wt+miR-NC group. (B) MiR-29a-3p expression was determined after transfection of NC, pcDNA3.1-H19 and si-H19. *$P < 0.05$ when compared with NC. (C) MiR-29b-3p was targeted by lncRNA H19, and luciferase activity of CFs was compared between pmiRGLO-H19-Wt+miR-29b-3p mimic group and pmiRGLO-H19-Wt+miR-NC group. *$P < 0.05$ when compared with pmiRGLO-H19-Wt+miR-NC group. (D) MiR-29b-3p expression was measured after transfection of NC, pcDNA3.1-H19, and si-H19. *$P < 0.05$ when compared with NC.
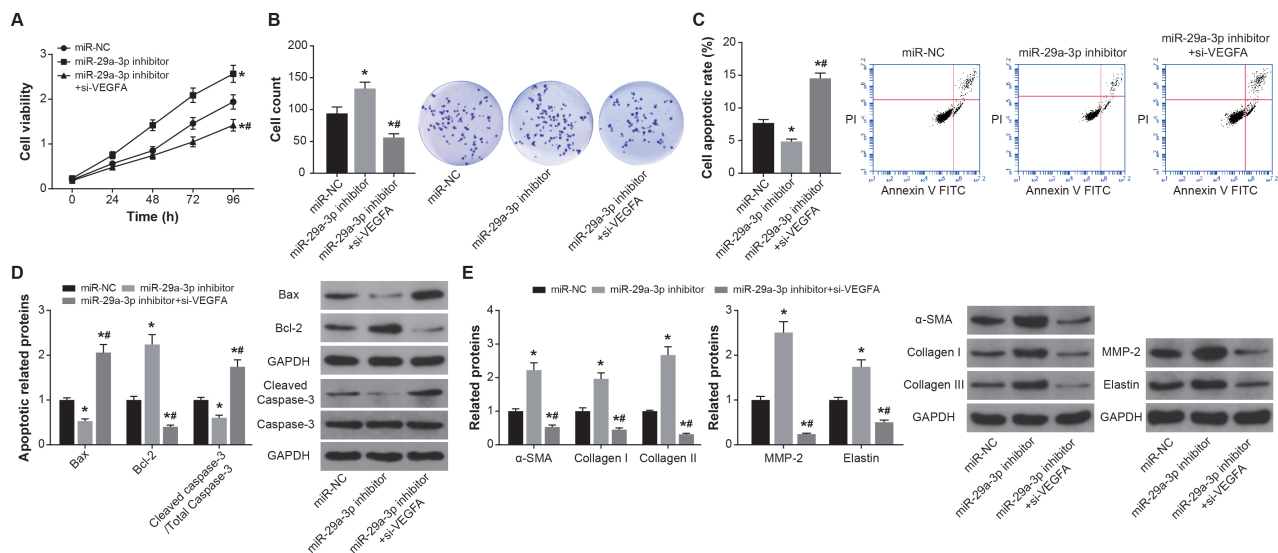

Fig. 7. Cell activities were impacted by miR-29a-3p and si-VEGFA in CFs. Viability (A), proliferation (B), apoptosis (C), apoptins (D), and ECM-related proteins (E) of CFs were evaluated among miR-NC, miR-29a-3p inhibitor, and miR-29a-3p inhibitor+si-VEGFA group. NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate. *$P < 0.05$ when compared with miR-NC, #$P < 0.05$ when compared with miR-29a-3p inhibitor.
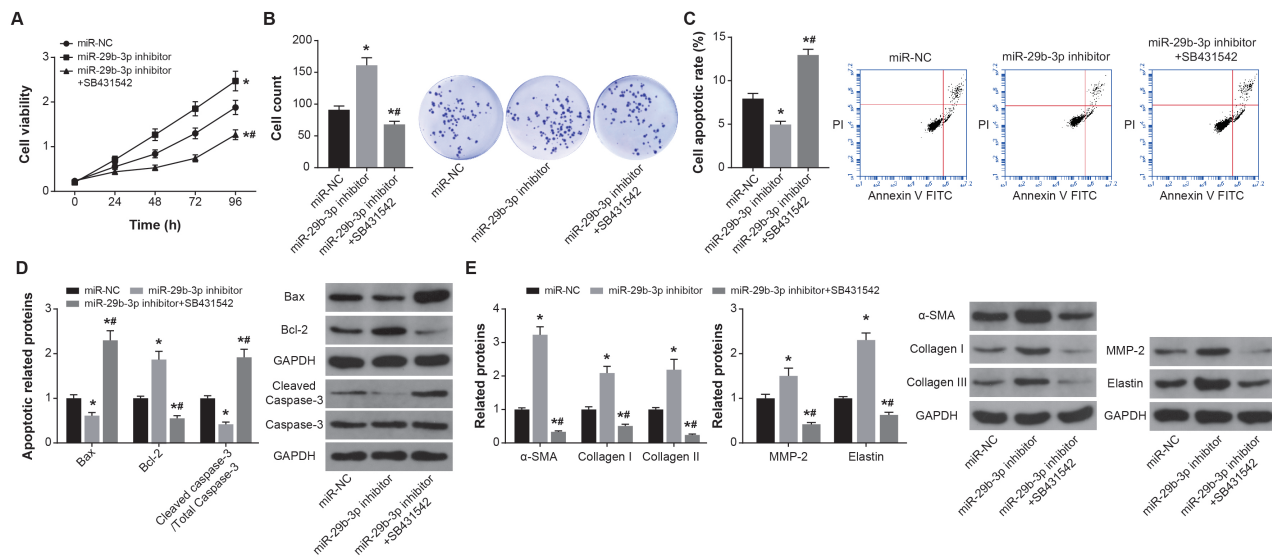
Fig. 8. Cell activities were regulated by miR-29b-3p and SB431542 in CFs. Viability (A), proliferation (B), apoptosis (C), apoptins (D), and ECM-related proteins (E) of CFs were evaluated among miR-NC, miR-29b-3p inhibitor and miR-29a-3p inhibitor+SB431542 group. NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate. *$P < 0.05$ when compared with miR-NC, #$P < 0.05$ when compared with miR-29b-3p inhibitor.

older (Fuster et al., 2001). AF typically leads to a reduction in heart function and the presence of arterial thromboembolism. The pathogenesis of AF is believed to relate to underlying factors including electrophysiological remodeling, structural remodeling, calcium homeostasis disorders, autonomic nervous system disorders, inflammation, and oxidative stress (Brundel et al., 2001; Dobrev and Nattel, 2008; Lee et al., 2006; Nattel et al., 2008; Neef et al., 2010). Notably, the formation of atrial fibrosis promotes the remodeling of atrial structure and AF onset. To date, however, the pathogenesis of AF has yet to be elucidated.

After physicochemical or biological stimulation, MF excessively proliferated, resulting in an overexpression of SMA and a massive deposition of ECM proteins, such as type I/III/IV collagen (Hennemeier et al., 2014; Mir et al., 2012). In this investigation, we suggested that H19 was capable of promoting MF multiplication and collagen production (Figs. 3 and 4), which provided a molecular explanation for H19 as a clinical biomarker for AF progression (Fig. 1A). Abundant miRNAs in human cardiac tissue were observed, including miR-29a/b, miR-27a/b, miR-30a/b/c, and miR-92a/b (Dobrev and Nattel, 2008), and appeared to play a crucial role in regulating cardiovascular function, including miR-195 and miR-9 in cardiac hypertrophy (van Rooij et al., 2006; Wang et al., 2010), miR-320 in myocardial infarction (Ren et al., 2009) and miR-590 in cardiac fibrosis (Shan et al., 2009). According to a newly identified regulatory network, crosstalk between lncRNAs and mRNAs occurs during competition for shared miRNA response elements. Based on this observation, lncRNAs may function as ceRNAs to sponge miRNAs, thereby modulating the distribution of miRNA molecules on their targets and imposing an additional level of post-transcriptional regulation. Our study suggests that low expression of miR-29a and miR-29b, resulting from sponging and negative reg-

ulation of lncRNA H19 (Fig. 6), promotes collagen production and multiplication of CFs (Figs. 4 and 5). The significant correlation between lncRNA H19 expression and miR-29a/29b expression among patients with AF (Fig. 1B) also showed that miR-29a/29b expression was clinically modified due to changes in lncRNA H19 expression.

As formerly documented, low miR-29 expression predisposes the onset of CF, myocardial infarction, and aortic constriction by targeting and up-regulating ECM genes, including collagen, metalloproteinases, elastin, and fibrillin (Abonnenc et al., 2013; van Rooij et al., 2008). Moreover, miR-29a could alleviate MF progression by up-regulating expression of RASSF1A (Tao et al., 2014b), thereby inhibiting development of fibrosis (Ramdas et al., 2013). Thus, it was demonstrated that a variety of AF-causing genes were modified by miR-29. Here, we observed that VEGFA was sponged by miR-29a in CFs, resulting in the activation of CF proliferation and collage release, which were inhibited by miR-29a (Fig. 7). In addition to CFs, VEGFA, an important mammalian growth factor (Koch et al., 2011), was also subjected to negative modulation of miR-29a in a human embryonic kidney cell line (i.e., HEK293) (Yang et al., 2013), suggesting that the miR-29a/VEGFA axis was shared by a variety of pathogenic mechanisms. Furthermore, impediment of VEGFA signaling clearly reduced the severity of liver fibrosis and slowed its increase (Yan et al., 2015). The HIF-1α-VEGF-ING-4 axis, the so-called angiogenesis axis, plays an important role in the pathogenesis of experimental pulmonary fibrosis and idiopathic pulmonary fibrosis (Smadja et al., 2014). Li et al. (2015) also found that the application of low molecular weight heparin reduced the expression of fibrosis-promoting factors in mice, such as VEGF, thereby attenuating the development of fibrosis. In summary, the miR-29a/VEGFA axis is vital for the role of lncRNA H19 in the development of AF. Furthermore, TGF-β

is considered to be a pivotal downstream molecule of miR-29b in regulating AF etiology. TGF-β, a major target for the treatment of fibrotic lesions (Edgley et al., 2012), may induce proliferation of myocardial fibroblasts, secretion of collagen, and differentiation of myocardial fibroblasts into myofibroblasts (Guo et al., 2018). It has been reported that blocking TGF-β/Smad3 signaling could restore and alleviate myocardial dysfunction and MF caused by miRNA-29b knockout (Zhang et al., 2014), which altogether suggests that TGF-β is pivotal in mediating the contribution of H19/miR-29b axis to AF progression.

In conclusion, lncRNA H19 contributes to the etiology of AF by dual regulation of the miR-29a/b-VEGFA/TGF-β axis, and this combination was an essential target in the development treatment strategies for AF. However, there were several shortcomings in the experimental design of this study. Firstly, patients with AF were not grouped according to severity of disease, so it was unclear whether the lncRNA H19-led miR-29a/b-VEGFA/TGF-β axis was altered to a different degree, with increasing severity of AF. Secondly, patients with AF who were included in the study were limited in terms of sample size and ethnicity. Therefore, it may not be appropriate to extrapolate the clinical conclusions from this study to populations of patients with AF. Thirdly, animal models of AF were not constructed to elucidate the role of the lncRNA H19-led miR-29a/b-VEGFA/TGF-β axis in AF progression. To increase the reliability of the underlying etiology of AF, additional, in-depth results from further studies will be needed.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

F.G., C.T., B.H., L.G., J.Z., Z.M., C.L., and Y.L. conceived and designed the experiments. F.G., C.T., B.H., and L.G. performed the experiments. J.Z. and Z.M. analyzed the data. C.L. and Y.L. drafted the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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