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Basic Science

Long Non-Coding RNA Dancr Affects Myocardial Fibrosis in Atrial Fibrillation Mice via the MicroRNA-146b-5p/Smad5 Axis

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Objectives: Atrial fibrillation (AF) is the most frequent arrhythmia, and myocardial fibrosis (MF) has a close association with atrial remodeling and leads to AF. This study aimed to explore the function of the long non-coding RNA (lncRNA) differentiation antagonizing non-protein coding RNA (Dancr)/microRNA (miR)-146b-5p/Smad5 axis on MF in AF mice.

Methods: AF mouse models were established. Overexpression Dancr lentivirus was injected into AF mice to increase Dancr expression in myocardial tissues. LncRNA Dancr, miR-146b-5p, and Smad5 expression levels and inflammatory factors (IL-18 and TNF-α) in the myocardial tissues were measured. MF was measured and the expression levels of MF-related genes (COL1A1, α-SMA, and FN1) were detected. In addition, *in vitro* HL-1 cell rapid pacing models were constructed, and after lncRNA Dancr and miR-146b-5p-related construct transfection, cell viability and cell apoptosis were determined.

Results: LncRNA Dancr up-regulation ameliorated MF in the AF mice, reduced IL-18 and TNF-α expression levels in myocardial tissues, and decreased COL1A1, α-SMA, and FN1 expression levels. The *in vitro* HL-1 cell rapid pacing models suggested that miR-146b-5p overexpression reversed the inhibitory effects of lncRNA Dancr overexpression on MF in HL-1 cells, and Smad5 interference reversed the ameliorative effects of miR-146b-5p interference on MF in HL-1 cells.

Conclusions: LncRNA Dancr can sponge miR-146b-5p to promote Smad5 expression, thereby delaying MF in AF mice.

Key Words: Atrial fibrillation • ceRNA • LncRNA Dancr • MicroRNA-146b-5p • Myocardial fibrosis • Smad5

INTRODUCTION

As the most frequent type of arrhythmia, atrial fibrillation (AF) has substantial related morbidity and mortality.[1] It is a progressive disorder, first being non-sustained and trigger activity-induced, then developing towards persistent AF due to changes in atrial myocardial substrate.[2] Myocardial fibrosis (MF) refers to a reactive remodeling process in response to myocardial injury.[3] MF alters the architecture of the myocardium, impairing cardiac function, facilitating arrhythmias, and affecting the clinical course and results of patients with heart failure.[4] AF is very common among the elderly and/or obese, and MF is related to AF.[5] MF is associated with atrial remodeling and results in AF. MF is considered to be the major reason for cardiovascular disorders and a pathological foundation of AF. Therefore, the underlying mechanisms of MF and AF progression should be fully probed to allow for novel AF treatments.[6]

Recently, long non-coding RNAs (lncRNAs) have emerged as vital modulators of cardiac diseases.[7] For example, it has been demonstrated that lncRNAs play a cru-

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Abbreviations

AF	Atrial fibrillation
ANOVA	Analysis of variance
Dancr	Differentiation antagonizing non-protein coding RNA
DAPI	4′,6-diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-phosphate dehydrogenase
GEO	Gene Expression Omnibus
lncRNAs	Long non-coding RNAs
M	Model
MF	Myocardial fibrosis
miRNA	microRNA
mRNAs	Messenger RNAs
MUT	Mutant
NC	Negative control
oe	Overexpression
PBS	Phosphate buffer saline
RIP	RNA immunoprecipitation
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TGF-β	Transforming growth factor beta
UTR	Untranslated region
WT	Wild-type

cial role in cardiac fibrosis[8] and in acute myocardial infarction progression.[9] LncRNA differentiation antagonizing non-protein coding RNA (Dancr) encoding human chromosome 4q12 was first identified as an inhibitor of epidermal cell differentiation.[10] LncRNA Dancr functions in the regulation of cell metabolism and diverse diseases. In addition, Dancr is a protective factor against calcification in acute myocardial infarction, stroke, and arterial calcification.[11] Recently, Dancr has been reported to alleviate hypoxia-induced injury in H9c2 cardiomyocytes by up-regulating hypoxia-inducible factor-1α.[12] However, no study has investigated the role of Dancr in myocardial fibrosis. Mechanistic analysis has indicated that Dancr can function as a microRNA (miRNA) sponge, stabilize messenger RNAs (mRNAs), and interact with proteins.[13] In addition, lncRNA Dancr overexpression has been shown to suppress apoptosis, enhance autophagy and protect cardiomyocytes from endoplasmic reticulum stress injury through sponging miR-6324.[14] miRNAs serve as negative regulators of protein translation by affecting mRNA stability, regulating various signaling pathways and cellular processes, and are linked to cell-to-cell communication.[15] MiRNAs have been used as AF fibrotic and electrical alteration biomarkers. Selective miRNA target therapy, through up-regulation by adenovirus transfection and/or miR down-regulation by antagomiR, can be used in the treatment of AF.[16] miR-146b is active in modulating immunomodulation, and miR-146b-5p has been reported to be up-regulated in not only the infarcted myocardium of mice but also the serum of patients with myocardial ischemia.[17] In addition, experiments conducted in human-induced pluripotent stem cell-derived atrial cardiomyocytes have revealed that miR-146b-5p suppression may be an efficient treatment method to prevent AF.[18] MiRNAs and circular lncRNAs, combined with lncRNAs, can affect transforming growth factor beta (TGF-β)/Smad signaling, which leads to cardiac fibrosis.[19] Smad signaling cascades have an important impact on cardiac fibrotic responses.[20] Smad5 is a member of the receptor-activated Smad that act as intracellular signal transducers in growth factor-beta superfamily transforming.[21] Nevertheless, the functions of the lncRNA Dancr/miR-146b-5p/Smad5 axis on MF in AF remain to be elucidated. Therefore, the aim of this research was to further explore the regulatory effects of lncRNA Dancr on MF in AF via mediating the miR-146b-5p/Smad5 axis, thus providing a novel direction for AF treatment.

MATERIALS AND METHODS

Ethics statement

All animal procedures and care were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the regulations of the Ethics Committee of our hospital.

Bioinformatics analysis

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds) was used to obtain differential expressions of lncRNAs in GSE10598 microarrays (*in vitro* mimic AF, *in vitro* HL-1 cell expression

profiles before and after rapid electrical stimulation), and differential analysis was carried out using the R language "limma" package. |log2(FoldChange)| > 1 and p < 0.05 were used as screening criteria for differential lncRNAs.

Establishment of AF mice models

Healthy male C57BL/6 mice (24.2 ± 2.1 g, 8 weeks old) were obtained from the Laboratory Animal Center of Shangdong University. The mice were kept at 20-26 °C, with a relative humidity of 40-70%, and under a 12: 12-h light-dark cycle (6:00 am to 6:00 pm), and had free access to water and food.

Wild-type (WT) mice were anesthetized by intraperitoneally injecting pentobarbital sodium (dose: 40 mg/kg) and supported by artificial respiration, with body temperature maintained at 37 °C and under full monitoring. Intracardiac pacing was conducted by inserting an 8-electrode catheter (1.1F, octapolar EP catheter, Scisense, London, Ontario, Canada) into the jugular vein and then advancing it into the right atrium. The atrial arrhythmia induction rate was measured by implementing 6-second pulses via the catheter electrodes utilizing the automatic stimulator of the data acquisition system. The cycle length of the first 6-second pulse was 40 ms, decreasing to a cycle length of 20 ms at a rate of 2 ms. Next, 50 μg/kg of carbachol was injected via the jugular vein. After 2 min, the same Burst pacing was applied to the animals. Mice intubated via the jugular vein but without atrial pacing were used as the sham-operated group. Successful AF induction was defined as a rapid irregular atrial rhythm lasting at least 1 second.[22,23]

***In vivo* lentivirus injection**

Prior to AF mouse model establishment, 10 μL of 1 × 10^8 TU lentiviral solution was injected into the mice using a microinjector (thoracic surgery was performed at 3-4 of the rib cage to expose the heart, and the mice were injected at different injection points into the right atrium). The animals were divided into the following groups (10 animals per group): the Sham group (sham-operated mice), the AF group (AF mice), the AF + overexpression (oe)-negative control (NC) group (AF mice injected with oe-NC lentivirus), and the AF + oe-Dancr group (AF mice injected with oe-Dancr lentivirus). After suturing the skin for 48 h, AF was induced for 5 consecutive weeks as described above. Subsequently, the mice in each group were euthanized. The myocardial tissues were collected and rinsed in ice-cold phosphate buffer saline (PBS), and some of the myocardial tissues were taken for reverse transcription quantitative polymerase chain reaction (RT-qPCR), while the rest was paraffin-embedded for subsequent pathological analysis.

All lentiviruses (3 μg pLenti-oe-Dancr, 1 μg pCMV-VSV-G, and 3 μg pCMV-Delta8.9) used in the experiments were transfected into 293T cells (ATCC, USA) using Lipofectamine 3000 reagent (Invitrogen, USA). After 20 h, the medium was replaced with 12 mL of medium containing 5% fetal bovine serum (FBS). After about 48 h, the virus-containing supernatants were collected, filtered through a 0.45 μm cellulose acetate filter (Merck Millipore, USA), and stored at -80 °C for experimental use.[24-26]

Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-18 levels in the mouse myocardial tissues and cell supernatants were measured by ELISA (R&D, USA). In brief, heart tissue homogenates or cell supernatants were placed into the plate wells, and after 30-min incubation at 37 °C, the liquid in the wells was removed and then the plate was washed. Next, the enzyme reagent was added, followed by incubation at 37 °C for 30 min. After that, the color developer was added and protected from light for 15 min, and then the termination solution was added. The optical density value was assessed by ELISA at 450 nm.[27]

Histopathological observation

The right ventricle was fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into sections of approximately 5 μm thickness. The sections were then used for Masson trichrome staining of myocardial collagen to observe the extent of MF. The results were observed using light microscopy, and Image-ProPlus6 software (Media Cybernetics, Rockville, USA) was used to calculate the areas of fibrosis.[28,29]

HL-1 atrial myocyte culture and rapid pacing

HL-1 atrial myocytes, obtained from adult mice atria (CL-0605, Wuhan Procell Life Science&Technology Co., Ltd., Wuhan, China) were preserved in complete Claycomb medium (Sigma, Zwijndrecht, The Netherlands),

followed by the addition of 10% FBS (PAA Laboratories GmbH, Austria), 100 U/mL penicillin (Gibco, Landsmeer, the Netherlands), 100 μg/mL streptomycin (Gibco), 4 mML glutamine (Gibco), 0.3 mML ascorbic acid (Sigma, St Louis, MO, USA), and 100 μM norepinephrine (Sigma). The HL-1 cells were cultured on glass coverslips coated with 0.02% gelatin (Sigma) and grown at 37 °C under 5% CO_2.

The HL-1 cells were rapidly electrically paced for 6 h at 1.5 V/cm, 4 ms, and 10 Hz using a C-Pace100TM culture pacemaker (IonOptix Corporation, Amsterdam, the Netherlands) (the Model group). Cells not paced for 6 h were used as baseline control (the control group). After 4.5 h, HL-1 cell medium was centrifuged at 1000 g for 5 min, and the supernatants were transferred to NIH/3T3 (CL-0171, Procell) embryonic fibroblasts. RT-qPCR was conducted to measure factors associated with fibroblast fibrosis induced by HL-1 cells.[30-32]

Cell transfection

HL-1 cells were transfected with oe-NC, oe-Dancr, oe-Dancr + miR-146b-5p mimic, inhibitor NC, miR-146b-5p inhibitor, and miR-146b-5p inhibitor + si-Smad5, respectively, using Lipofectamine 3000. The plasmids, mimic, inhibitor, and siRNA used above were provided by GenePharma (Shanghai, China). After transfection for 48 h, transfection effects were verified using RT-qPCR. The HL-1 cells were then induced by rapid electrical pacing using a pacemaker.[33]

Cell viability detection

The trypan blue dye exclusion method was conducted to determine cell viability. Cells were trypsinized and resuspended in a culture medium. The cell suspension was then mixed with the same volume of 0.4% trypan blue solution (Gendepot, Barker, TX, USA) and cultured for 1 min. Cell viability was evaluated by counting the number of live and dead cells with a cell counter under a light microscope (Olympus, Inc, Center Valley, PA).[32]

Cell apoptosis analysis

The HL-1 cells treated in each group were rinsed 3 times using PBS, fixed in 4% paraformaldehyde for 15 min at 37 °C, stained with a one-step TUNEL Apoptosis Detection Kit (Green Fluorescence) (Beyotime Biotechnology, Shanghai, China) for 1 h at 37 °C, and stained with 4′,6-Diamidino-2-phenylindole (DAPI) at room temperature for 10 min. Five fields of view were randomly selected to observe apoptotic cells under a fluorescent microscope. The percentage of apoptotic cells was calculated as the ratio of the number of TUNEL-positive nuclei to the number of DAPI-stained cells.

In vitro apoptosis assays were performed using a PE Annexin V Apoptosis Assay Kit I (559763, BD Biosciences, USA). Briefly, differently treated HL-1 cells were trypsinized in 1×10^6 cells/pipe and rinsed twice with cold buffer. HL-1 cells were incubated with 5 μL PE-Annexin V and 5 μL 7-AAD for 15 min at room temperature in the dark and assessed by BD FACSCelesta flow cytometry. Data were analyzed using Flowjo software (BD Biosciences).[34]

Dual luciferase reporter gene assay

The 3′ untranslated region (UTR) of full-length lncRNA Dancr and smad5 were amplified and cloned into pmirGLO (Promega Corporation, Madison, WI, USA) luciferase vectors to construct Dancr-WT and smad5-WT vectors. Next, the corresponding binding sites were mutated to construct Dancr-mutant (MUT) and smad5-MUT vectors using the renilla luciferase-expressing pRL-TK vector (Promega) as an internal reference. The correctly sequenced recombinant luciferase reporter plasmids WT and MUT were cotransfected into HL-1 cells with miR-146b-5p mimic or mimic NC, respectively. The cells were collected and lysed 48 h after transfection, and relative luciferase activity was tested using a dual luciferase report gene assay (Promega), which was then normalized to renin luciferase activity in accordance with the manufacturer's protocol.[35]

RNA immunoprecipitation (RIP) assay

RIP analysis was conducted utilizing an EZ-Magna RIP Kit (Millipore, USA). First, HL-1 cells were collected and lysed in a complete RIP lysis buffer. Next, cell extracts were cultured with RIP buffer containing magnetic beads conjugated with anti-Ago2 antibody (1:50, ab32381, Abcam, UK). Samples were cultured with proteinase K and shaken to digest the proteins, and immunoprecipitated RNA was extracted. Subsequently, the RNA concentration was assessed using a nanodrop spectrophotometer, and the purified RNA was subjected to real-time PCR analysis.

RNA pull-down assay

HL-1 cells were transfected with biotin-labeled RNA biotin-labeled miR-146b-5p (bio-miR-146b-5p-WT and bio-miR-146b-5p-MUT) and NC (bio-NC) (50 nM each), respectively. After transfection for 48 h, the cells were collected and rinsed with PBS. The cells were subsequently cultured with the specific cell lysate for 10 min, and then 50 mL of sample cell lysate was dispensed. The residual lysate was incubated with streptavidin magnetic beads (LSKMAGT, Sigma) pre-coated with RNase-free and yeast tRNA [Thermo Fisher Scientific (CHINA) Co., Ltd., Shanghai, China] for 3 h at 4 °C, and rinsed twice with cold lysate, three times with low salt buffer, and once with high salt buffer. RNA was isolated and RT-qPCR was conducted to detect the expression levels of Dancr in the bio-miR-146b-5p (WT/MUT) or bio-NC pull-down samples.[36]

RT-qPCR

Total RNA was isolated using Trizol reagent (Beyotime), and for lncRNA and mRNA, RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara, Dalian, China). Next, PCR amplification was implemented using TB Green® Premix Ex Taq™ II (Takara). To detect miR-146b-5p expression levels, reverse transcription and qPCR were implemented using an miRcute plus miRNA first-strand cDNA synthesis kit (Tiangen Biotech, Beijing, China) and miRcute Plus miRNA qPCR detection kit (Tiangen Biotech). Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as the internal reference for lncRNA and mRNA, and U6 was used as an internal reference for miRNA. The relative expression levels of the genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.[36-38]

Western blot analysis

Radio-immunoprecipitation assay lysate containing protease inhibitors (Boster, Wuhan, China) was used to extract total proteins, and then protein concentrations were tested using a bicinchoninic acid protein quantification kit (Boster). Equal amounts of proteins were isolated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis gels, transferred to polyvinylidene fluoride membranes, and sealed with 5% bovine serum albumin at room temperature for 2 h to block non-specific binding. The membranes were then cultured at 4 °C overnight with diluted primary antibodies: Smad5 (1:1000, ab40771, Abcam) and GAPDH (1:1000, ab9485, Abcam). After that, the membranes were cultured with HRP-labelled goat anti-rabbit secondary antibody (ab 205718; 1:2000; Abcam) at room temperature. Signals were detected using an electrochemiluminescence assay (Amersham), and densitometry (Syngene, GenetooLs) was used for quantification, with GAPDH as an endogenous reference.

Statistics

All analyses were conducted using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation. Comparisons between two groups were carried out using the unpaired t-test, while comparisons among multiple groups were conducted using one-way analysis of variance, followed by Tukey's post hoc tests. $p < 0.05$ was an indicator of a significant difference.

RESULTS

Low expression of Dancr in the AF mice

To explore which lncRNAs regulate MF in AF, we an-

Table 1. Primer sequences for genes used in RT-qPCR assay

Genes	Primer sequences (5′-3′)
Dancr	F: AAGTGAGGGCCATGTGAGTTT
	R: CTAGGGCAGTGGGACATGAAG
miR-146b-5p	F: TGAGAACTGAATTCCATAGGCT
	R: Universal primers
Smad5	F: GAGCCATCACGAGCTAAAACC
	R: ACTGGAGGTAAGACTGGACTCT
COL1A1	F: GGTCTTGGTGGTTTTGTATTCG
	R: AACAGTCGCTTCACCTACAGC
α-SMA	F: CCCAGACATCAGGGAGTAATGG
	R: TCTATCGGATACTTCAGCGTCA
FN1	F: TTCAAGTGTGATCCCCATGAAG
	R: CAGGTCTACGGCAGTTGTCA
U6	F: GCTTCGGCAGCACATATACTAA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: AAGCCCATCACCATCTTCCAGGAG
	R: AGCCCTTCCACAATGCCAAAG

Dancr, Differentiation antagonizing non-protein coding RNA; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-146b-5p, microRNA-146b-5p; R, reverse; Smad5, SMAD family member 5.

alyzed the differential expressions of lncRNAs in GSE 10598 microarrays, which indicated that six lncRNAs were obtained, namely Snhg12, Snhg7, Gas5, Dancr, Pvt1, and Sngh3, and all displayed down-regulation (Figure 1A). We further established AF mouse models to examine the expressions of these six lncRNAs in the myocardial tissues of AF mice, and found that Dancr was the most significantly down-regulated (Figure 1B). This suggested that Dancr may be a key factor in the regulation of AF.

Phylogenetic analysis processed using the UCSC Genome Browser (http://genome.ucsc.edu/) demonstrated that Dancr is highly conserved in mammals, indicating that Dancr possesses an important function (Figure 1C). To verify whether Dancr could regulate MF in AF, AF mice were injected with oe-Dancr lentivirus, after which the expression of Dancr was found to be increased (Figure 1D). ELISA results demonstrated elevated levels of inflammatory factors IL-18 and TNF-α in the myocardial tissues of AF mice, and that these levels decreased in the mice injected with oe-Dancr (Figure 1E). RT-qPCR results demonstrated that the mRNA expression levels of COL1A1, α-SMA, and FN1, the genes associated with central myofibrosis, were increased in AF mice, and that these levels were also reduced in the mice injected with oe-Dancr (Figure 1F).

Masson staining revealed that in normal mice, myocardial fibers were normal in morphology and orderly arranged, whereas myocardial cells in AF mice had a disordered fiber arrangement and showed myocardial cell swelling, and the area of fibrosis was increased. In AF mice after oe-Dancr injection, myocardial fibers were abnormally arranged and infiltrated by inflammatory cells in the center of myocardial tissues, and the area of fibrosis was reduced (Figure 1G).

The experimental results suggested that the overexpression of Dancr could improve MF in AF mice.

Dancr competitively sponged miR-146b-5p

To further investigate Dancr regulatory mechanisms, we identified possible binding sites between Dancr and miR-146b-5p via the Starbase bioinformatics analysis website (https://starbase.sysu.edu.cn/) (Figure 2A). It has previously been indicated that miR-146b-5p can promote AF fibrosis.[18] We further validated this by dual luciferase reporter gene assay, and the findings indicated that in HL-1 cells transfected with miR-146b-5p mimic, Dancr-WT luciferase activity was inhibited, however, the

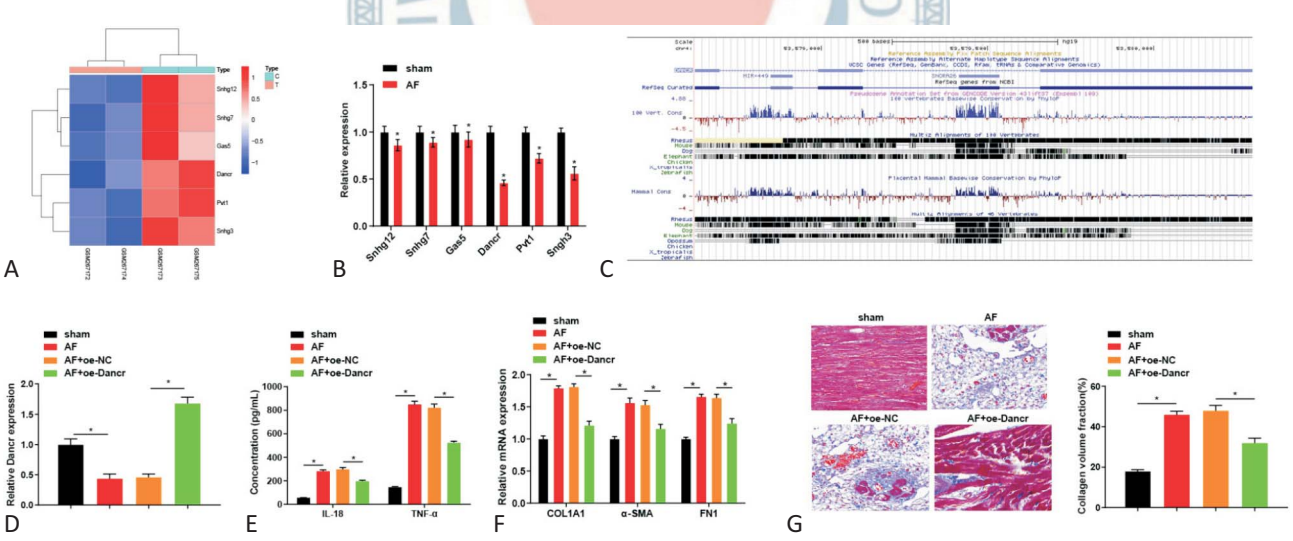

Figure 1. *Differentiation antagonizing non-protein coding RNA (Dancr) expression levels in atrial fibrillation (AF) mice. (A) Heat map of differential-expressed lncRNAs in GSE10598 microarrays, with red representing up-regulation and blue representing down-regulation. (B) The expression levels of lncRNAs in the myocardial tissues of sham-operated mice and AF mice after overexpression (oe)-Dancr treatment were tested by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (C) UCSC Genome Browser was utilized to display a graphical view of Dancr's multi-species comparisons, with blue peaks and red peaks indicating conservation scores. (D) Dancr expression levels in the myocardial tissues of sham-operated mice and AF mice after oe-Dancr treatment were assessed by RT-qPCR. (E) IL-18 and TNF-α expression levels in myocardial tissues of sham-operated mice and AF mice after oe-Dancr treatment were assessed by enzyme-linked immunosorbent assay (ELISA). (F) The expression levels of MF-related genesin sham-operated mice and AF mice after oe-Dancr treatment were assessed by RT-qPCR. (G) Myocardial cell fibrosis in sham-operated mice and AF mice after oe-Dancr treatment was measured by Masson trichrome staining. * $p < 0.05$, $n = 10$.*

luciferase activity of the Dancr-MUT reporter plasmid had no impact (Figure 2B).

To verify the endogenous binding of miR-146b-5p to Dancr at the cellular level an RIP assay was performed (Figure 2C). The results demonstrated that IP with anti-ago2 antibody indicated a strong interaction between miR-146b-5p and Dancr, suggesting that Dancr may be present in the miR-146b-5p RISC complex. Furthermore, the results of an RNA pull-down assay showed that Bio-miR-146b-5p-WT could enrich more Dancr, while Bio-miR-146b-5p-MUT displayed no significant change (Figure 2D), indicating that miR-146b-5p was sequence-specific in recognizing Dancr. We further examined miR-146b-5p expression levels in AF mice, and the findings revealed increased expression levels in myocardial tissues of AF mice, but decreased expression levels in AF mice after oe-Dancr treatment (Figure 2E). Pearson correlation analysis showed that miR-146b-5p had a negative correlation with Dancr expression (Figure 2F). In conclusion, these results revealed that miR-146b-5p could be sponged by Dancr.

MiR-146b-5p reversed the inhibitory effects of Dancr on MF

To further investigate the regulatory role of Dancr on miR-146b-5p, HL-1 cells were transfected with oe-Dancr or miR-146b-5p mimic, and rapid pacing was used to induce *in vitro* models. We found that the expression of Dancr was decreased whereas the expression level of miR-146b-5p was elevated in the Model (M) group compared with that in the Control group. Overexpressing miR-146b-5p reversed the inhibitory effects of Dancr overexpression on miR-146b-5p (Figure 3A). We further assessed the physiological status of HL-1 cells, and found that the M group had lower cell viability and increased apoptosis in comparison with the Control group, whereas the M + oe-Dancr group displayed increased viability and decreased apoptosis compared with the M + oe-NC group (Figure 3B-D). The levels of inflammatory cytokines IL-18 and TNF-α in the cell supernatants and the expressions of MF-related genes COL1A1, α-SMA, and FN1 in fibroblasts after co-culture with HL-1 cell supernatants were further examined, and the results showed that when compared with those in the Control group, IL-18 and TNF-α contents and the mRNA expression levels of COL1A1, α-SMA, and FN1 were higher in the M group, while these factors were all decreased in the M + oe-Dancr group compared to those in the M + oe-NC group (Figure 3E-F). In addition, miR-146b-5p overex-

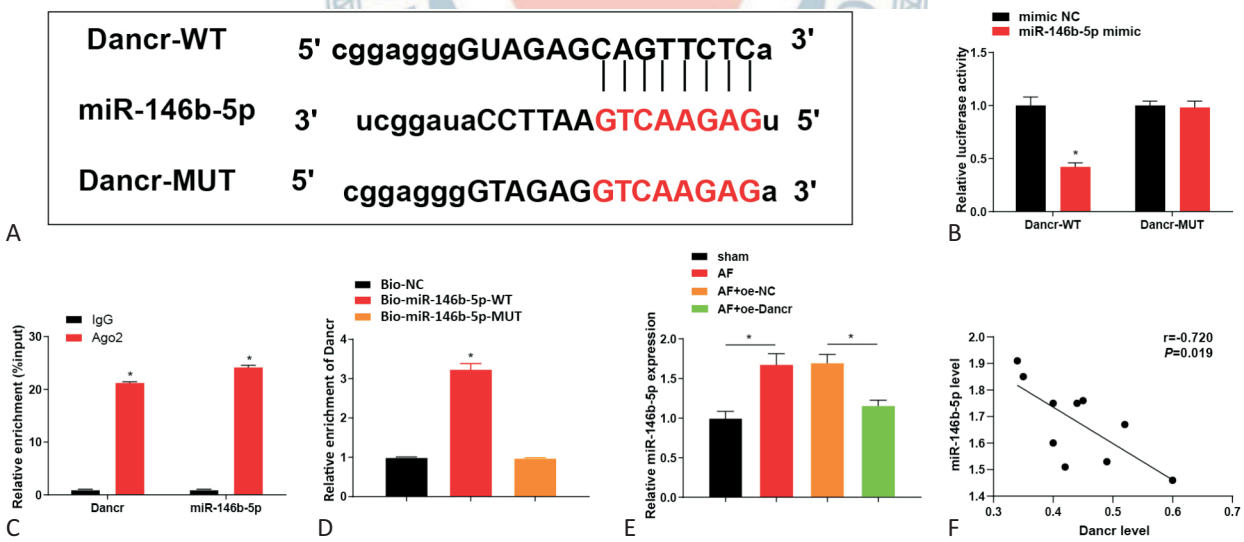

Figure 2. *There are binding sites between differentiation antagonizing non-protein coding RNA (Dancr) and microRNA (miR)-146b-5p. (A) Starbase website predicted the binding sites between Dancr and miR-146b-5p. (B) Binding sites and mutation sites between Dancr and miR-146b-5p; the luciferase activity of Dancr-wild-type (WT)/mutant (MUT) in HL-1 cells after miR-146b-5p mimic transfection was analyzed utilizing dual luciferase reporter gene assay. (C) The relationship between Dancr and miR-146b-5p in HL-1 cells was detected by RNA immunoprecipitation (RIP) assay. (D) Dancr expression levels were tested by biotin-coupled miR-146b-5p pull-down assay. (E) miR-146b-5p expression in myocardial tissues of atrial fibrillation (AF) mice after overexpression (oe)-Dancr injection was assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR), n = 10. (F) The correlation between levels of Dancr and expression of miR-146b-5p in myocardial tissues of AF mice was evaluated using Pearson correlation test, n = 10; * p < 0.05.*

pression reversed the delaying effects of Dancr on MF in HL-1 cells (Figure 3B-F). The results showed that miR-146b-5p reversed the inhibitory effects of Dancr on MF in HL-1 cells *in vitro*.

MiR-146b-5p had a targeting relationship with Samd5

Through the Starbase bioinformatics analysis website (https://starbase.sysu.edu.cn/), we found possible binding sites between Smad5 and miR-146b-5p (Figure 4A). A dual luciferase reporter gene assay was conducted and further verified that the luciferase activity of Smad5-WT in HL-1 cells after miR-146b-5p treatment was inhibited, but that Smad5-MUT had no significant impact (Figure 4B).

We then further examined the Smad5 levels in AF mice, and the results demonstrated that Smad5 expression levels were decreased in tissues of AF mice, but increased in the mice injected with oe-Dancr (Figure 4C-D). Pearson correlation analysis showed that Smad5 was negatively correlated to miR-146b-5p (Figure 4E). Further *in vitro* validation revealed that Smad5 expression was reduced in the M group in comparison with that in the Control group, and that the overexpression of miR-146b-5p could reverse the promoting effects of Dancr overexpression on Smad5 (Figure 4F). These findings demonstrated that Smad5 was a downstream target gene of miR-146b-5p.

MiR-146b-5p up-regulated Smad5 to promote MF in HL-1 cells *in vitro*

To further investigate whether Dancr could affect AF MF via the miR-146b-5p/Smad5 axis, we set up the M + inhibitor NC, the M + miR-146b-5p inhibitor, and the M + miR-146b-5p inhibitor + si-Smad5 groups. The results revealed that the expression of miR-146b-5p was reduced and the level of Smad5 mRNA was increased in

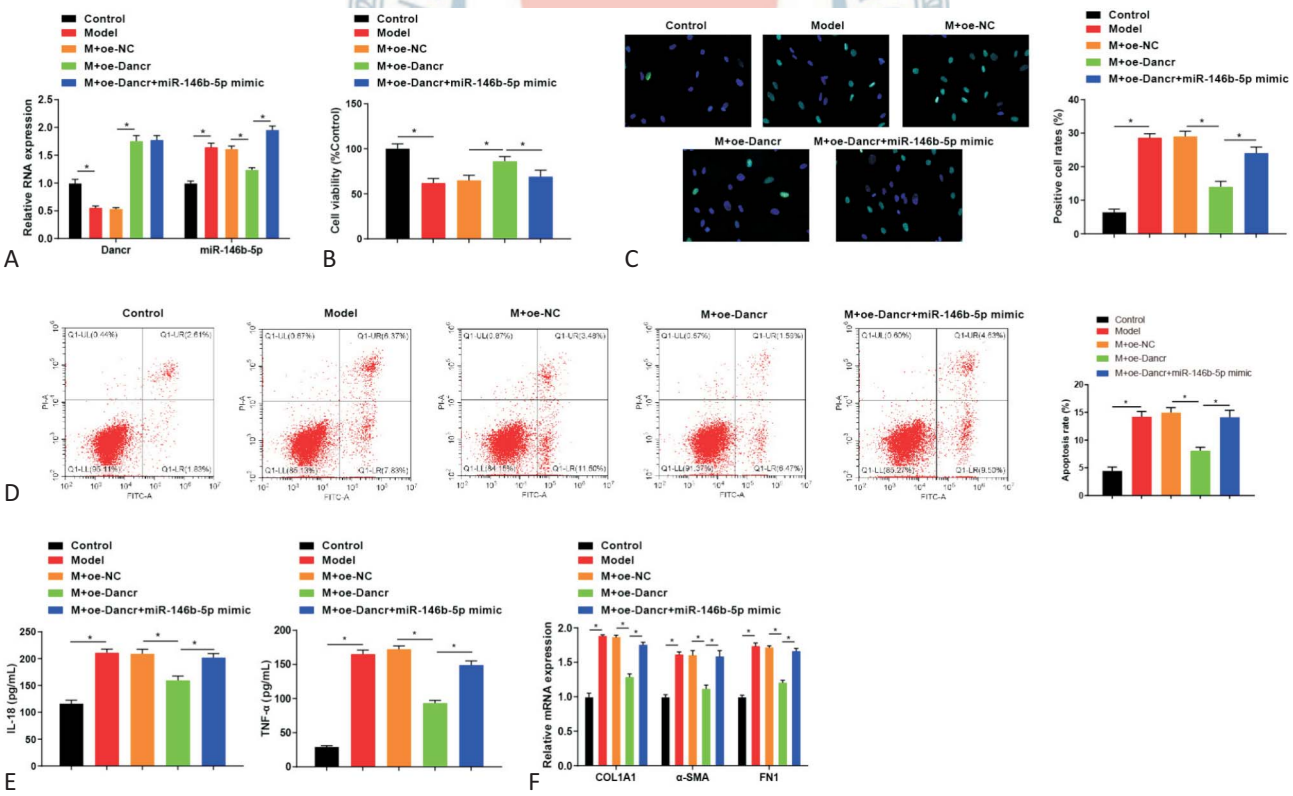

Figure 3. *MicroRNA (miR)-146b-5p reverses the impact of differentiation antagonizing non-protein coding RNA (Dancr) on myocardial fibrosis (MF) in HL-1 cells in vitro. (A) The expression levels of Dancr and miR-146b-5p after overexpression (oe)-Dancr and miR-146b-5p mimic transfection were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B) HL-1 cell viability after oe-Dancr and miR-146b-5p mimic transfection was determined by Trypan Blue staining. (C) HL-1 cell apoptosis after oe-Dancr and miR-146b-5p mimic transfection was assessed by TUNEL assay. (D) Apoptosis of HL-1 cells and miR-146b-5p mimic transfection was assessed by flow cytometry. (E) Inflammatory factors IL-18 and TNF-α contents in HL-1 cell supernatants after oe-Dancr and miR-146b-5p mimic transfection were tested by enzyme-linked immunosorbent assay (ELISA). (F) The expression levels of MF-related genes in fibroblasts after co-culture with HL-1 cell supernatants after oe-Dancr and miR-146b-5p mimic transfection were assessed by RT-qPCR. * $p < 0.05$. M referred to Model.*

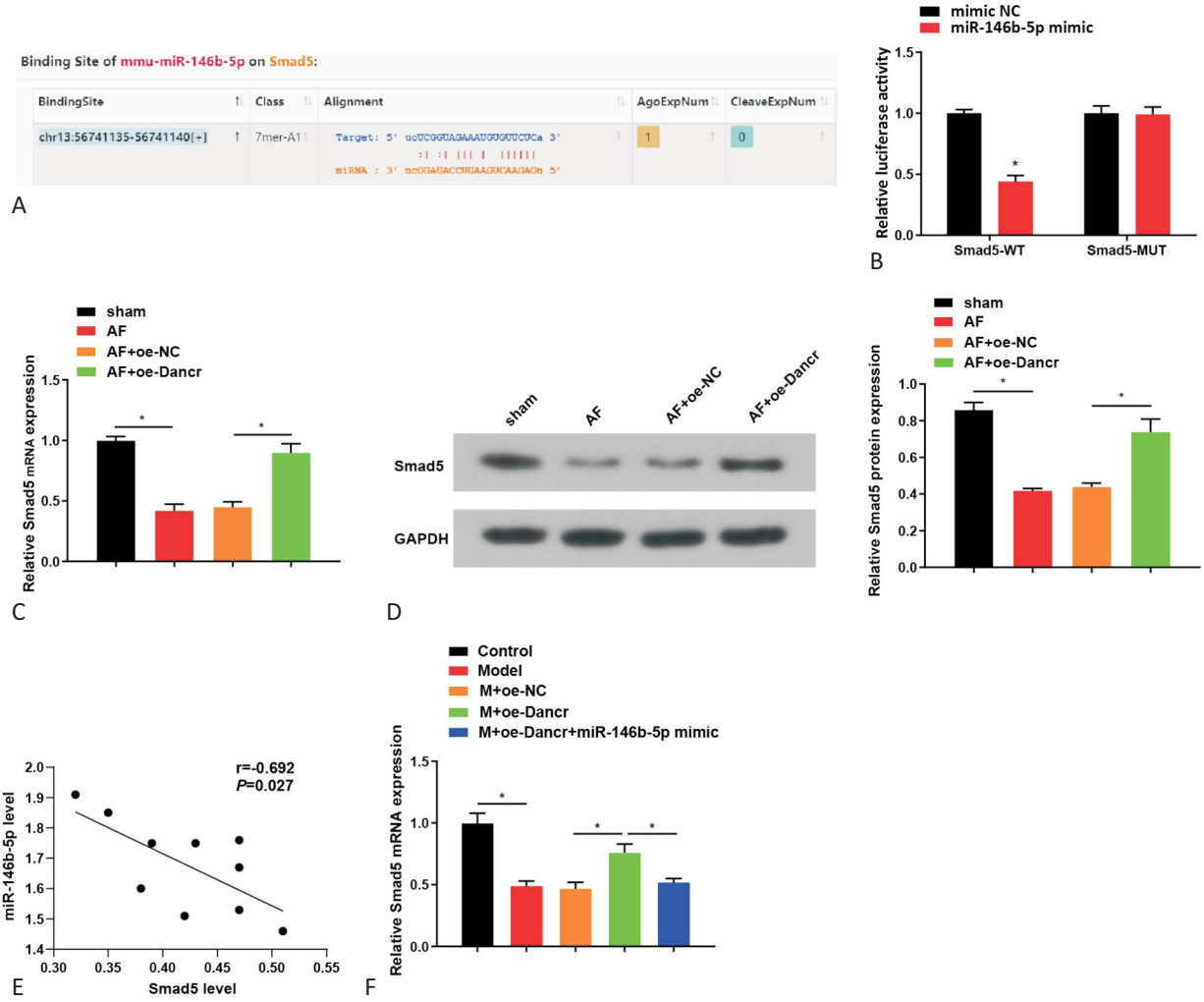

Figure 4. *Smad5 is a downstream target gene of microRNA (miR)-146b-5p. (A) The binding sites between Smad5 and miR-146b-5p were predicted by Starbase website and mutated. (B) Smad5-wild-type (WT)/mutant (MUT) luciferase activity in HL-1 cells after miR-146b-5p mimic treatment were analyzed by dual luciferase reporter gene assay. (C) Smad5 messenger RNA (mRNA) expression in myocardial tissues of sham-operated mice and atrial fibrillation (AF) mice after overexpression (oe)-differentiation antagonizing non-protein coding RNA (Dancr) treatment was tested by reverse transcription quantitative polymerase chain reaction (RT-qPCR), n = 10. (D) Smad5 protein expression in the myocardial tissues of sham-operated mice and AF mice after oe-Dancer was measured by western blot, n = 10. (E) The correlation between miR-146b-5p levels and Smad5 mRNA expression in myocardial tissues of AF mice was analyzed using Pearson correlation test. (F) Smad5 mRNA expression in HL-1 cells after oe-Dancr and miR-146b-5p mimic transfection were assessed by RT-qPCR. * $p < 0.05$.*

the M + miR-146b-5p inhibitor group compared with the M + inhibitor NC group. In addition, compared with the M + miR-146b-5p inhibitor group, Smad5 mRNA level was reduced in the M + miR-146b-5p inhibitor + si-Smad5 group (Figure 5A).

Further assessment of the physiological status of HL-1 cells showed that compared with the M + inhibitor-NC group, the M + miR-146b-5p inhibitor group had increased cell viability, decreased apoptosis, reduced contents of IL-18 and TNF-α in cell supernatants, as well as reduced mRNA expression levels of COL1A1, α-SMA, and FN1. In addition, si-Smad5 could reverse the delaying effects of miR-146b-5p interference on AF (Figure 5B-F). These results suggested that Dancr delayed MF in HL-1 cells *in vitro* by modulating the miR-146b-5p/Smad5 axis.

DISCUSSION

AF is a highly prevalent arrhythmia in clinical prac-

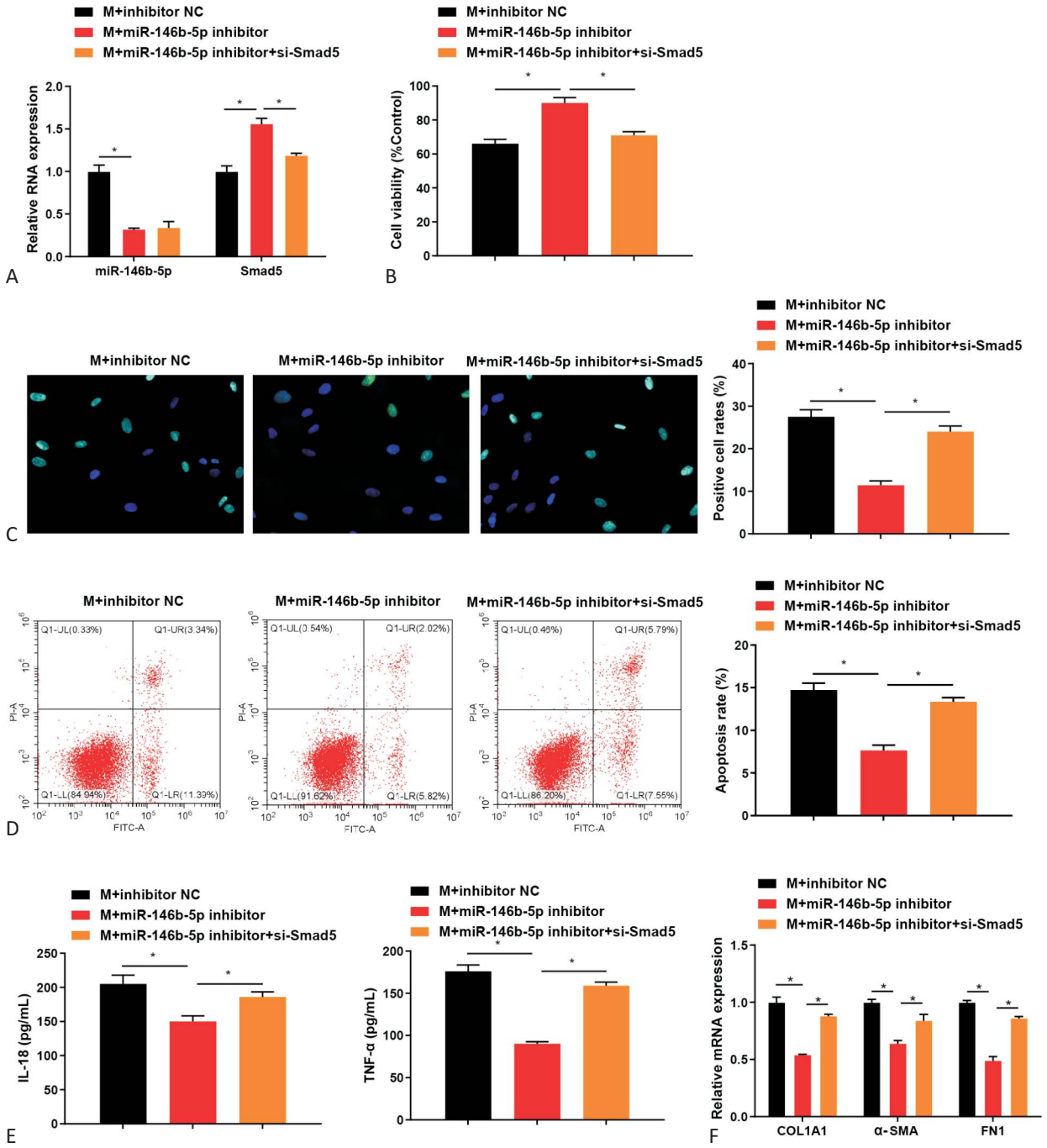

Figure 5. *The microRNA (miR)-146b-5p/Smad5 axis affects myocardial fibrosis (MF) in HL-1 cells in vitro. (A) miR-146b-5p and Smad5 messenger RNA (mRNA) expression levels after miR-146b-5p inhibitor and si-Smad5 transfection were assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B) HL-1 cell viability after miR-146b-5p inhibitor and si-Smad5 transfection was evaluated by Trypan Blue staining. (C) HL-1 cell apoptosis after miR-146b-5p inhibitor and si-Smad5 transfection was determined by TUNEL assay. (D) Apoptosis of HL-1 cells after miR-146b-5p inhibitor and si-Smad5 transfection was tested by flow cytometry. (E) Inflammatory factors IL-18 and TNF-α contents in HL-1 cell supernatants after miR-146b-5p inhibitor and si-Smad5 transfection was measured by enzyme-linked immunosorbent assay (ELISA). (F) MF-related genes expression levels in fibroblasts after co-culture with HL-1 cell supernatants after miR-146b-5p inhibitor and si-Smad5 transfection were tested by RT-qPCR. * $p < 0.05$.*

tice, and it is closely related to a higher risk of stroke, peripheral embolism, and death.[39] MF is a frequent pathological manifestation during the terminal stage of multiple cardiovascular disorders, often resulting in se-

vere cardiac dysfunction and even death.[40,41] In this research, we investigated a novel mechanism for MF in AF by evaluating the role of the lncRNA Dancr/miR-146b-5p/Smad5 axis.

As previously reported, lncRNAs are emerging as important regulators of cardiac development and disease,[42] including during cardiac progression, cardiac hypertrophy, MF, and myocardial ischemic injury.[43] LncRNAs perform a crucial role in cardiovascular diseases, and lncRNA Dancr alleviates cardiomyocyte damage.[14] LncRNA Dancr has been associated with the development of atherosclerosis.[44] In addition, lncRNAs have been reported to act as key regulators of acute myocardial infarction, and the knockdown of Dancr has been shown to decrease myocardium apoptosis in mice with the left anterior descending artery ligated.[45] In our study, we found a low expression of Dancr in AF mice, and that Dancr overexpression could improve MF in AF mice. Dancr is considered to potentially be able to improve the regeneration of tissues. Mechanically, Dancr, as a cytoplasmic lncRNA, can sponge corresponding miRNAs or interact with a variety of proteins.[11] Moreover, the suppression of DANCR has been shown to inhibit inflammation and proliferation, and promote chondrocyte apoptosis.[46] This is consistent with our findings.

MiRNAs exert crucial roles not only in physiology and cardiovascular system development but also in cardiovascular disorder development and progression. Recent research on tissue-specific miRNAs has indicated a role in structural and electrical remodeling in AF.[47] miRNAs are considered to be a potential method for the treatment of AF.[48] Many miRNA genes found in many animals possess the functions of modulating cell proliferation, cell death, and hematopoiesis, and they have even been shown to be involved in cardiovascular disease.[49] Emerging evidence has shown that identifying miRNAs and mRNAs may reveal potential and novel molecular modulation networks, offering a better understanding of the molecular basis of AF remodeling. Additionally, miR-146b-5p may intracellularly mediate maladaptive remodeling in atrial fibrosis in AF.[50] As previously reported, miR-146b-5p inhibition may be a novel treatment method for cardiac fibrotic remodeling and dysfunction after MI.[17] miR-146b-5p may also be a potential biomarker and treatment factor for hepatic fibrosis, and miR-146b-5p is up-regulated during hepatic fibrosis progression.[51] This is consistent with our experimental results that miR-146b-5p expression levels were increased in the myocardial tissues of AF mice.

We also found that miR-146b-5p could be sponged by Dancr. Some articles have demonstrated a targeting relationship between miRNAs and Dancr. Dancr has been reported to compete with miR-335-5p binding, and lncRNA Dancr has a close association with atherosclerosis progression by targeting miR-335-5p, indicating that Dancr may be a potential detective predictor and target in the treatment of atherosclerosis.[52] In addition, Dancr has been shown to regulate osteoarthritis chondrocyte survival by acting as a competitive endogenous RNA for miR-216a-5p.[46] Some articles have also demonstrated a targeting relationship between miRNAs and Smad5. For instance, a previous study reported that Smad5 is a target gene of miR-122-5p,[53] and another study reported that Smad5 is also a target of miR-124-3p and is correlated with miR-124-3p-mediated biological effects.[54] SMAD5 expression knockdown has also been correlated with increased apoptosis of human granulosa cells.[55] In our study, we identified possible binding sites between Smad5 and miR-146b-5p, verified a targeting relationship between miR-146b-5p with Smad5, and found that Smad5 was a downstream target gene of miR-146b-5p. Furthermore, we found that miR-146b-5p up-regulated Smad5 to promote MF in HL-1 cells *in vitro*, and that Dancr delayed MF in HL-1 cells *in vitro* via the miR-146b-5p/Smad5 axis.

CONCLUSIONS

In conclusion, this research provides evidence that lncRNA Dancr competitively sponges miR-146b-5p to promote Smad5 expression, thus delaying MF in AF mice. This study provides a novel strategy and distinct theoretical references for the treatment of MF in AF by highlighting the regulatory roles of lncRNA Dancr, miR-146b-5p, and Smad5. However, we used HL-1 cells instead of human-induced pluripotent stem cell-derived atrial cardiomyocytes for the *in vitro* study, which is the main limitation of this study, and further research is needed to obtain more precise data for a convincing conclusion. Additionally, in our *in vitro* fibrosis study, we investigated the effects of the Dancr/miR-146b-5p/Smad5 axis on

myocardial fibrosis by co-culturing treated HL-1 cardiomyocyte medium supernatants with fibroblasts. Previous studies have demonstrated that cardiomyocytes may regulate fibroblast fibrosis through a paracrine mechanism.[56,57] However, due to financial limitations, we could not conduct specific in-depth exploration in this study.

DECLARATION OF CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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