IL-6-miR-210 Suppresses Regulatory T Cell Function and Promotes Atrial Fibrosis by Targeting Foxp3

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The aim of this study was to explore the role of IL-6-miR-210 in the regulation of Tregs function and atrial fibrosis in atrial fibrillation (AF). The levels of interleukin (IL)-6 and IL-10 in AF patients were detected by using ELISA. Proportions of Treg cells were detected by fluorescence activated cell sorting analysis in AF patients. The expression of Foxp3, α-SMA, collagen I and collagen III were determined by western blot. The atrial mechanocytes were authenticated by vimentin immunostaining. The expression of miR-210 was performed by quantitative real-time polymerase chain reaction (qRT-PCR). TargetScan was used to predict potential targets of miR-210. The cardiomyocyte transverse sections in AF model group were observed by H&E staining. The myocardial filaments were observed by masson staining. The level of IL-6 was highly increased while the level of IL-10 (Tregs) was significantly decreased in AF patients as compared to normal control subjects, and IL-6 suppressed Tregs function and promoted the expression of α-SMA, collagen I and collagen III. Furthermore, miR-210 regulated Tregs function by targeting Foxp3, and IL-6 promoted expression of miR-210 via regulating hypoxia inducible factor-1α (HIF-1α). IL-6-miR-210 suppresses regulatory T cell function and promotes atrial fibrosis by targeting Foxp3.

Keywords: Foxp3, interleukin-6, miR-210, Treg cell function

INTRODUCTION

Atrial fibrillation (AF) is one of the most common and clinically significant arrhythmias (Chugh et al., 2014), and it is associated with increased morbidity and mortality (Thrall et al., 2006). The mechanisms of AF are multifarious and connected with electrical and structural remodelling in the atria and ventricles. The development and progression of atrial fibrosis are marked by structural remodelling and are believed to be the cause for AF immortalization (Dzeshka et al., 2015). AF is considered to be a significant public health issue; unfortunately, there is no current effective means of AF prevention. Therefore, it is valuable to investigate the mechanism of occurrence and development of AF.

CD4+ CD25+ regulatory T cells (Tregs) have been recognized as having an essential role in maintaining peripheral tolerance as well as preventing and limiting autoimmune and chronic inflammatory diseases (Wang et al., 2016). In recent years, an increasing number of studies have focused on the effects of Tregs on tissue fibrosis in various diseases. Claassen et al. (2010) investigated the correlation between CD4+FoxP3+Tregs and liver fibrosis induced by inflammation, and the authors confirmed that the highly activated Tregs located in the inflamed liver were responsible for the reduction in fibrosis. Another study reported that Tregs alleviate cardiac

hypertrophy and myocardial fibrosis in Angiotensin II-induced hypertensive mice (Matsumoto et al., 2011). Our previous studies demonstrated that the imbalance of Th17/Treg cells contributes to the underlying mechanisms of AF. Here, we further investigated the influence of Treg function on atrial fibrosis from a molecular mechanistic perspective.

MicroRNAs (miRNAs) are endogenous, noncoding RNAs composed of 21 to 23 nucleotides. A growing body of research demonstrates that miRNAs are involved in immune responses (Huang et al., 2015; Salama et al., 2014), especially in the regulation of Tregs (Chong et al., 2008; Lu et al., 2010). Foxp3 was identified as the key transcription factor in Tregs; Foxp3 determines the lineage of Tregs and is also the major regulatory gene for the development of Tregs (Hori and Sakaguchi, 2004). Studies have shown that miR-210 targets Foxp3 (Zhao et al., 2014). Interleukin-6 (IL-6) has been shown to be of particular importance in regulating the balance between Treg and Th17 cells, since IL-6 decreases the number of Treg cells found in the T cell population (Samson et al., 2012). These findings prompt us to hypothesize that IL-6 might contribute to the mechanisms of atrial fibrosis through the miR-210 pathway.

In this study, we investigated the role and mechanism of IL-6-miR-210 in the regulation of Treg function and atrial fibrosis. We found that IL-6-miR-210 suppresses regulatory T cell function and promotes atrial fibrosis by targeting Foxp3. This finding provides novel insight into the molecular mechanisms of AF.

MATERIALS AND METHODS

Study population
Sixteen patients with AF were recruited from the department of cardiology and the inpatient ward at The First Affiliated Hospital of Zheng Zhou University. The diagnosis of AF subtypes was determined by electrocardiography or continuous monitoring during the first 72 days after surgery. In the control group, 14 sinus rhythm subjects were recruited from The First Affiliated Hospital of Zheng Zhou University. The exclusion criteria were as follows: history of AF or other arrhythmia, use of any anti-arrhythmic medications (other than β-blockers), presence of permanent pacemakers, past or planned Cox maze procedure, more than moderate mitral valve disease, mitral valve prosthesis, congenital cardiac abnormalities, need for emergency surgery, systemic inflammatory or terminal illness, human immunodeficiency virus (HIV) infection, or lipodystrophy. At the time of sample collection, none of the patients had received corticosteroid or non-steroidal anti-inflammatory drug treatment. This study was approved by the human ethics committee of The First Affiliated Hospital of Zheng Zhou University, and written informed consent was obtained from all participants.

Blood sampling
Peripheral whole blood samples were collected from patients in a fasting state in the morning, and then they were placed in EDTA anticoagulant tube for 30 min, and centrifuged at 3,000 r/min for 5 min. The upper layer serum was collected and stored at −80°C until analysed.

Cell isolation, culture, and transfection
Cardiac tissue was removed from mice under aseptic conditions, and primary atrial fibroblasts were isolated by using enzyme digestion.

CD4+ T cells were isolated from the spleen tissues of mice. The purification of CD4+ T lymphocytes was conducted by an automated magnetic cell sorting kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Then, CD4+ T cells were transfected with negative control, miR-210 mimic or miR-210 inhibitor (Ambion, USA) using T cell Nucleofector Kits and hypoxia inducible factor-1α (HIF-1α) siRNA (Selleck Chemicals, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were collected for further analysis.

Tregs were isolated using a CD4+ CD25+ regulatory T-cell isolation kit (Miltenyi Biotec, USA). Isolated Tregs were then cultured with anti-CD3/CD28 antibodies (10 mg/ml) in 96-well plates in Dulbecco's modified Eagle medium containing 10% foetal calf serum and the antibiotics streptomycin and penicillin. The medium was changed 24 h later and then every 2 days during the 4-day culture period. The purity of the CD4+CD25+Foxp3+Tregs was assessed by flow cytometry using anti-mouse Foxp3-APC (eBioscience, USA) and anti-mouse CD25-PE (eBioscience) antibodies. The purity of the expanded Tregs used for adoptive transfer was greater than 90%. Cells were resuspended in phosphate-buffered saline (PBS) and then injected into an animal model of atrial fibrosis.

ELISA
The supernatant from peripheral whole blood samples and atrium dextrum were measured with IL-6 and IL-10 ELISA kits (Abcam, USA) according to the manufacturer's instructions. The concentration was calculated according to the corresponding optical density value.

Fluorescence activated cell sorting (FACS) analysis
Cell surface markers (FITC-conjugated CD4) and intracellular cytokines (PE-conjugated Foxp3) (BD Pharmingen™; BD Biosciences, USA) were stained and measured. FACS measurements were performed on a FACS Canto II (BD Biosciences), and data were analysed using FlowJo software (Tree Star, USA).

Western blotting
Total proteins were extracted from CD4+ T cells using RIPA lysis buffer (Beyotime Biotechnology, China) and were quantified with a BCA kit (Beyotime Biotechnology). Equal volumes of protein were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking in PBS with 5% non-fat milk and 0.05% Tween-20 for 1 h at room temperature, the membrane was incubated overnight at 4°C with the corresponding primary antibodies: Foxp3 (1:500; Abcam), α-SMA (1:500; Abcam), collagen I (1:500; Abcam), collagen III (1:500; Abcam), and HIF-1α (1:500; Abcam). Then, the membrane was incubated for 2 h with secondary antibodies conjugated with horseradish peroxidase at room temperature, and an ECL kit was used to detect immunoreactive bands according to the manufacturer's instruc-

tions (Thermo Scientific, USA). A fluorescent western blotting detection system was used.

Vimentin immunostaining
Identification of primary atrial mechanocytes was performed by vimentin immunostaining. First, mechanocytes were washed 2 to 3 times with PBS, fixed with 4% polyformaldehyde for 20 minutes, rinsed with PBS three times, treated with 0.5% Triton-100 X at room temperature for 20 min, and rinsed with PBS three times for 2 min each time. Cells were blocked with 1% bovine serum albumin overnight and then they were incubated with the antibody (anti-Vimentin antibody; Sigma, USA) at a 1:100 dilution at 4°C overnight. They were then rinsed with PBS three times for 2 min each time, and the next antibody was added at a 1:100 dilution (sheep anti-rabbit labelled with fluorescent dye Alexa Flour 488 and the antirat IgG antibody; Sigma). The cells were incubated with the fluorescent antibodies for 30 min at 37°C, and PBS was used to rinse the cells three times, 2 min each time; then, the cells were incubated with 1:1,000 Drap5 for 15 min to dye the nuclei, and laser scanning confocal microscopy was used to observe and photograph the cells.

Quantitative real-time polymerase chain reaction (qRT-PCR)
The mRNA expression of miR-210 and Foxp3 was detected by using qRT-PCR. Total RNA was extracted from the CD4+ T cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The Power SYBR Green RT-PCR Kit (Invitrogen) and the Bio-RAD CFX96 Real-Time System (Bio-RAD, USA) were used for qRT-PCR analysis. Data were normalized to the reference gene GAPDH for each cDNA sample.

Dual luciferase reporter assay
The 3′-UTR of the human gene Foxp3 was amplified from human cDNA. The wild-type fragment containing the predicted miR-210 binding site and its mutant fragment were designed to carry sites for SacI (5′ end) and XbaI (3′ end) at their ends, and they were obtained from the 3′-UTR of Foxp3. Amplicons were cleaved with SacI and XbaI and inserted between the SacI and XbaI cleavage sites of the pmir-GLO vector (Promega, USA). CD4+ T cells were selected on the basis of low endogenous miRNA expression. Cells were seeded in 24-well plates. When the cells reached 70% to 80% confluence, 800 ng of wild-type or mutant reporter and miR-210 mimic inhibitor (Ambion) were co-transfected into CD4+ T cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured in cell lysates using a dual-luciferase reporter system.

H&E staining and Masson staining
The myocardial tissue was harvested and fixed in 4% neutral paraformaldehyde and embedded in paraffin. The tissue specimens were sectioned at a thickness of 5 μm and conventionally stained with H&E for histological examination. Fibrosis was semi-quantitatively assessed with the Image-Pro plus analysis system following Masson staining.

Animal experiments
Ten-week-old male C57Bl6 mice were obtained from The Experimental Animal Centre of Zheng Zhou University. Ang II (2 μg/kg/min; Solarbio, China) was dissolved in 80 μl sterile saline and loaded into a Mini-Osmotic Pump (ALZET, USA). For pump insertion, mice were anaesthetized with isoflurane, and the upper back was cleaned with betadine/70% ethanol. A 1.0 cm skin incision was made in the upper back, and then a Mini-Osmotic Pump was implanted under the skin. In the control group, 80 μl of normal saline was added to the Mini-Osmotic Pump, and the other treatments were the same as the experimental group. Three weeks later, the mice were anaesthetized, and cardiac tissues were removed. Then, the weights of the hearts were calculated. H&E staining (transverse sections of heart) was performed to observe cardiac tissue morphology and cell size. Myocardial interstitial fibrosis was detected by Masson staining, and the expression of α-SMA, collagen I and collagen III was detected by western blot. All experiments were approved by The First Affiliated Hospital of Zheng Zhou University Animal Ethics Committee (2018-KY-386).

Statistical analysis
All data were analysed with SPSS (ver. 16.0; SPSS, USA). Data are presented as the mean ± SD. Student's t-test was used to analyse differences between two groups. One-way ANOVA was used to determine the multi-sample analysis. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

The levels of IL-6 and IL-10 are negatively correlated in patients with AF
To identify the relationship between IL-6 expression and IL-10 expression in patients with AF, the levels of IL-6 and IL-10 were measured by ELISA. Compared to normal control subjects, the level of IL-6 was highly increased in peripheral whole blood and right atrial tissue of AF patients, while the level of IL-10 was significantly decreased (Figs. 1A and 1B). Foxp3 is a specific transcription factor of Treg cells. The FACS results showed that compared to the normal control, the proportions of Treg (Foxp3-positive) cells were markedly lower in peripheral whole blood and right atrial tissue of AF patients (Figs. 1C and 1D). These results suggest that the levels of IL-6 and IL-10 are negatively correlated.

IL-6 suppresses the function of Tregs and promotes fibrosis of mechanocytes
To explore the effects of IL-6 on Tregs and mechanocytes in atrial tissue, CD4+ T cells were isolated from spleen tissues of normal mice and were first stimulated by treatment with IL-6 or PBS. Western blot results showed that the protein level of Foxp3 in the IL-6 group was significantly lower than that in the control group (Fig. 2A). In addition, the expression of IL-10 was markedly downregulated in the IL-6 group (Fig. 2B). Similarly, stimulation with IL-6 significantly decreased the levels of Treg cells compared to the control treatment (Fig. 2C). Atrial mechanocytes were isolated from the atrial tissue of mice and authenticated by vimentin immunostaining (Fig.
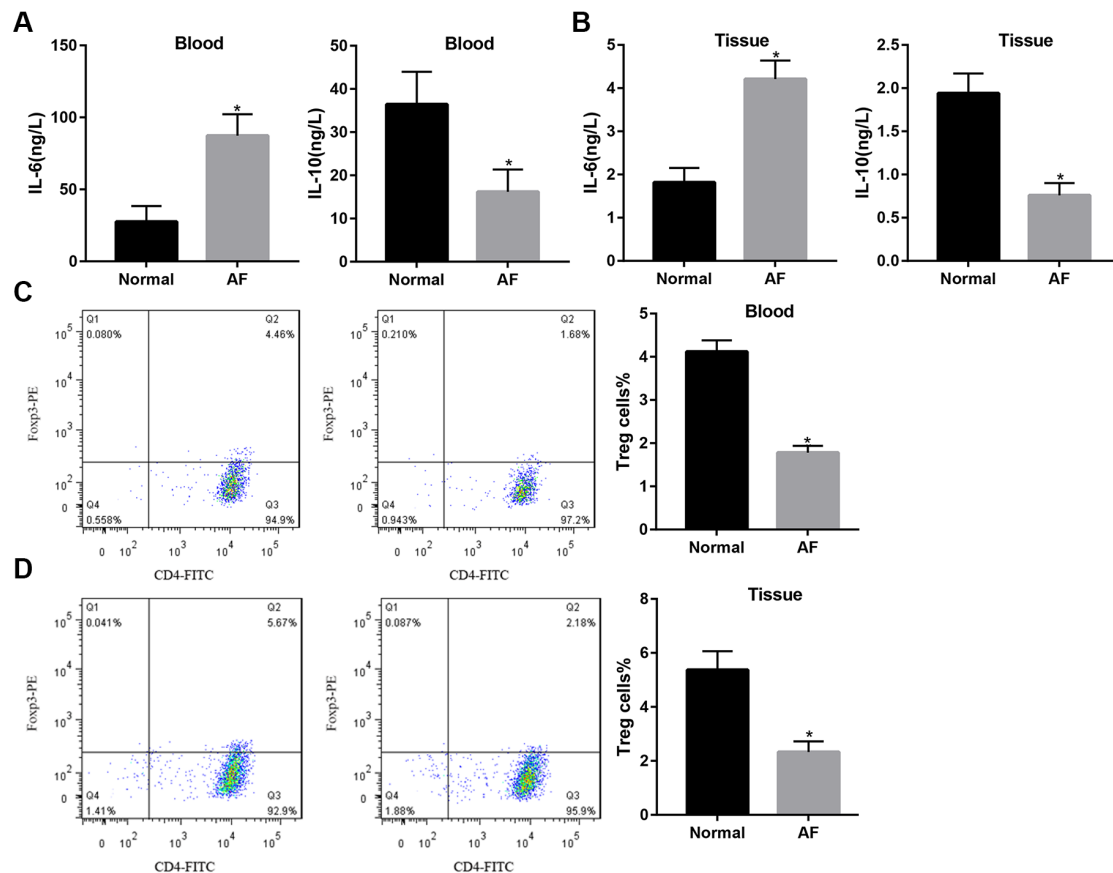
Fig. 1. Negative correlation between the levels of IL-6 and IL-10 in patients with AF. (A) ELISA results of IL-6 and IL-10 in peripheral whole blood of AF patients and normal subjects. (B) ELISA results of IL-6 and IL-10 in right atrial tissue of AF patients and normal subjects. (C) FACS analysis of Treg cells (FoxP3) in peripheral whole blood of AF patients and normal subjects. (D) FACS analysis of Treg cells (FoxP3) in right atrial tissue of AF patients and normal subjects. $*P < 0.05$ vs nomal controls.

2D). Ang II is an important marker for AF and atrial fibrosis (Kishore et al., 2014), and high expression of Ang II promotes the structural remodelling of the atria via boosting collagen synthesis (Ashikaga et al., 2006). α-SMA is a surface marker of cardiac myofibroblasts, and it reflects the state of cardiac fibroblast proliferation and transformation (Creemers and van Rooij, 2016). Additionally, cardiac myofibroblasts mainly secrete collagen I, collagen III and other collagen proteins (Ai et al., 2015). Hence, CD4+ T cells treated with IL-6, IL-10 and PBS were co-cultured with atrial mechanocytes induced by Ang II, and a western blot assay was performed to detect the expression of α-SMA, collagen I and collagen III. As shown in Fig. 2E, the expression of α-SMA, collagen I and collagen III significantly increased in the CD4+ T + IL-6 group compared with the CD4+ T + PBS group, while it dramatically decreased in the CD4+ T + IL-10 group. From these results, it is clear that IL-6 represses Treg function and promotes mechanocyte fibrosis.

IL-6 inhibits Treg cell function by regulating miR-210 by targeting Foxp3

To reveal the molecular mechanism by which IL-6 represses Treg activation, qRT-PCR was performed to determine miR-210 expression. Compared with normal control subjects, AF patients exhibited higher expression levels of miR-210 in serum and right atrial tissue (Fig. 3A), and miR-210 was upregulated in CD4+ T cells stimulated by IL-6 (Fig. 3B). The miRNA target gene prediction site TargetScan was used to predict potential targets of miR-210. Among the candidates, we found a highly conservative and specific combination sequence between miR-210 and the Foxp3 3'UTR; further, the miR-210 mimic significantly repressed luciferase activity when co-transfected with a reporter containing the WT Foxp3 3′UTR but not the MT Foxp3 3′UTR (Fig. 3C). CD4+ T cells were transfected with a miR-210 mimic and an inhibitor and then tested for expression of Foxp3. As shown in Figs. 3D and 3E, the mRNA and protein levels of Foxp3 were significantly lower in the miR-210 mimic group but markedly higher in the miR-210 inhibitor group compared to the control group. Further, transfected CD4+ T cells were stimulated by IL-6 and PBS, and the results indicated that the IL-6+miR-210 inhibitor significantly promoted the expression of IL-10 and Foxp3 compared to control (Figs. 3F and 3G). Finally, the CD4+ T cells transfected with the miR-210 mimic and inhibitor were co-cultured with mechanocytes induced by Ang II. In comparison with the control treatment, the Ang
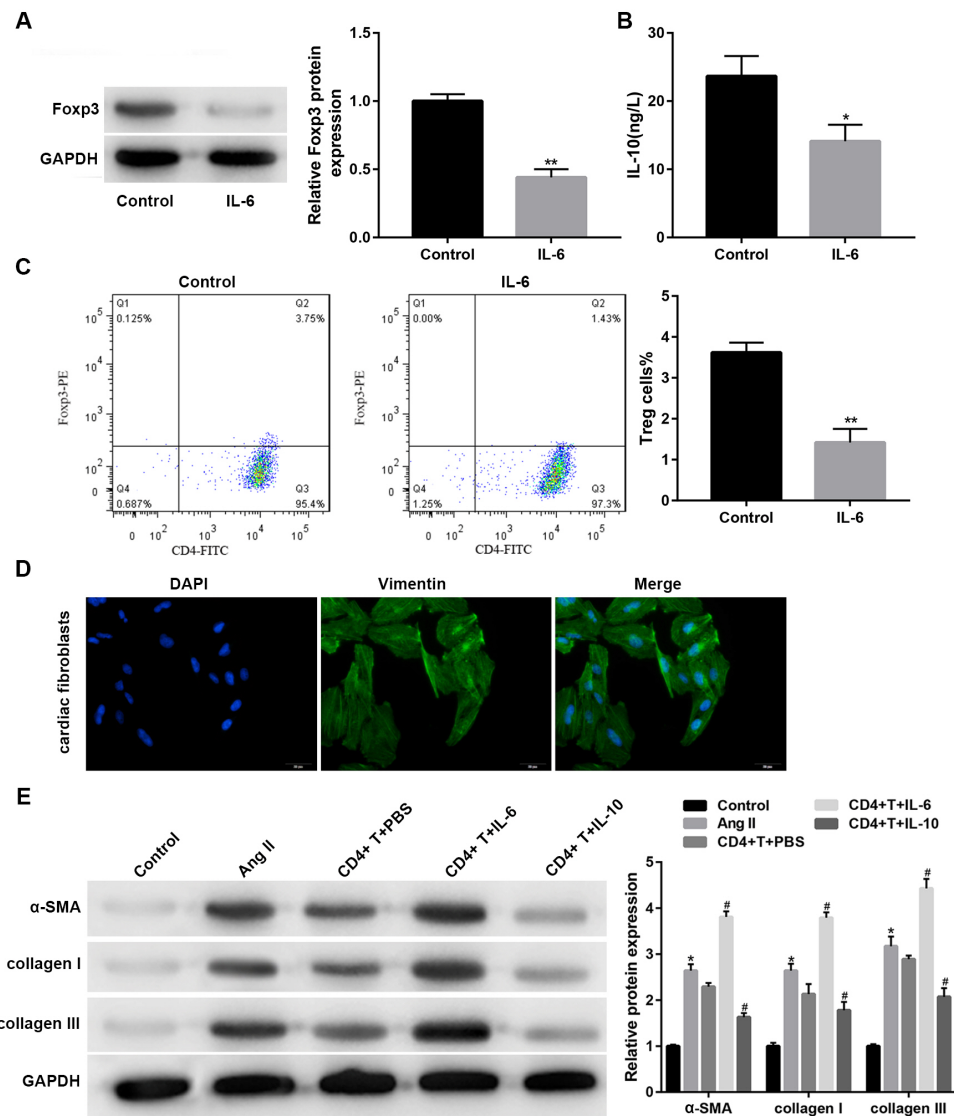
Fig. 2. IL-6 supresses the function of Tregs and promotes fibrosis of mechanocyte. (A) Western blot result of Foxp3 in CD4+ T cells. (B) ELISA results of IL-10 in CD4+ T cells. (C) FACS analysis of Treg cells (FoxP3) in CD4+ T cells. (D) Vimentin immunostaining of mechanocyte in atrial tissue of mice. (E) Western blot result of α-SMA, collagen I and collagen III in CD4+ T cells co-cultured with atrium mechanocyte. *$P < 0.05$, **$P < 0.01$ vs control; #$P < 0.05$ vs CD4+ T + PBS.

II+miR-210 mimic significantly promoted the expression of α-SMA, collagen I and collagen III, while the Ang II+miR-210 inhibitor dramatically inhibited their expression (Fig. 3H). Thus, IL-6 blocks the function of Tregs by regulating miR-210 by targeting Foxp3.

IL-6 promotes the expression of miR-210 via regulating HIF-1α

MiR-210 is a hypoxia-specific miRNA whose expression is regulated by the hypoxic induction factor HIF-1α. To explore the mechanism by which IL-6 regulates miR-210, the expression of HIF-1α was measured by western blot. Figs. 4A and 4B show that compared to healthy control patients, HIF-1α was significantly upregulated in the right atrial tissue of AF

patients and in CD4+ T cells stimulated by IL-6. Moreover, CD4+ T cells were transfected with HIF-1α siRNA and stimulated by IL-6. We found that IL-6+si-HIF-1α significantly suppressed the expression of miR-210, while promoting the expression of IL-10 compared to control treatment (Figs. 4C and 4D). Additionally, IL-6+si-HIF-1α significantly upregulated the levels of Treg cells and FoxP3 compared to control treatment (Figs. 4E and 4F). Finally, the CD4+ T cells transfected with HIF-1α siRNA were co-cultured with mechanocytes induced by Ang II. In comparison with the control group, the Ang II+si-HIF-1α group showed marked suppression of the expression of α-SMA, collagen I and collagen III (Fig. 4G). Taken together, these results suggest that IL-6 mediates the expression of miR-210 by regulating HIF-1α expression.
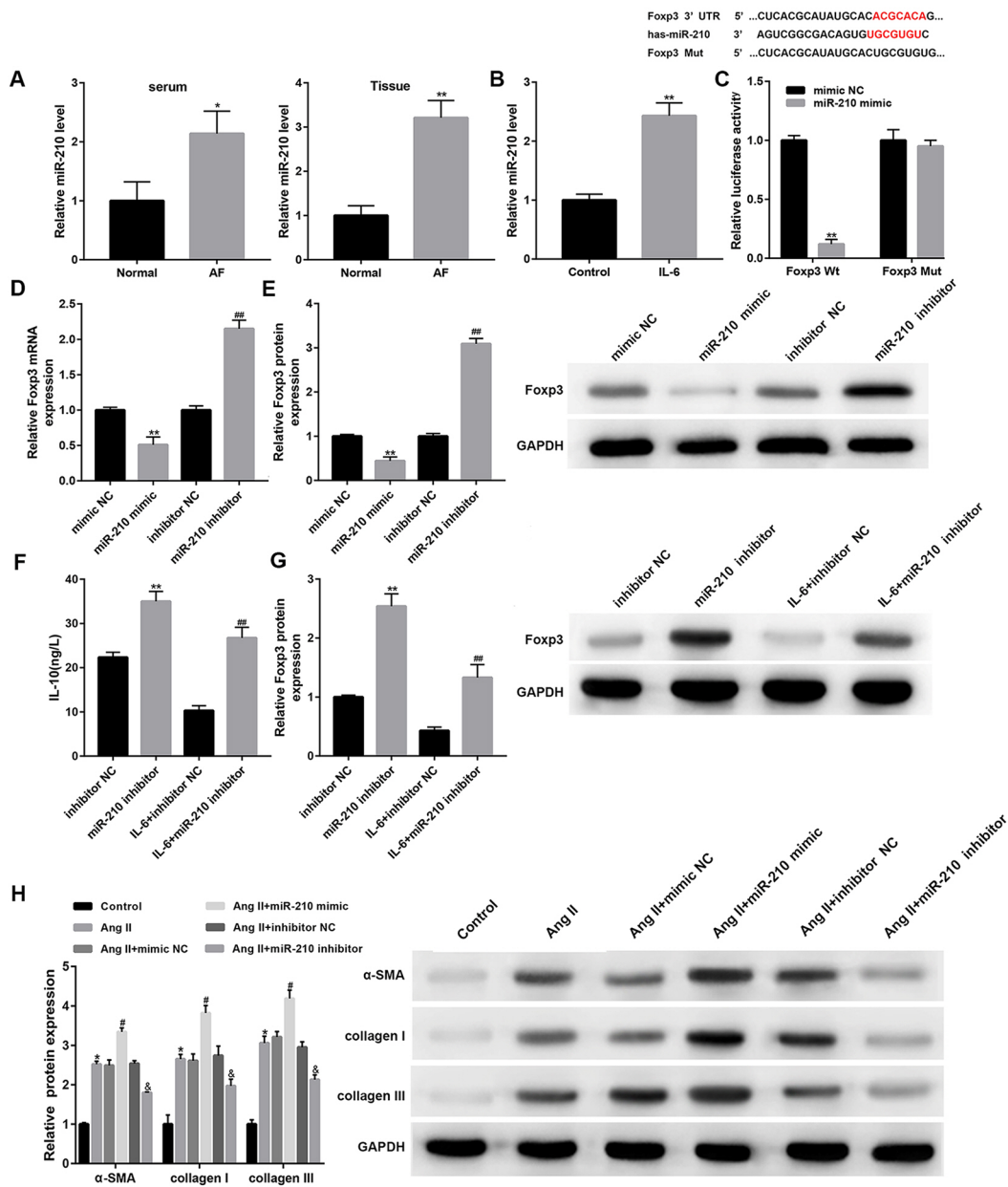
Fig. 3. IL-6 inhibits Treg cell function via regulating miR-210 by targeting Foxp3. (A) The qRT-PCR result of miR-210 in serum and right atrial tissue of AF patients. (B) The mRNA level of miR-210 in CD4+ T cells stimulated by IL-6 and PBS. (C) Schematic of the putative miR-210 target site in human Foxp3 3′-UTR and the seven mutated nucleotides are colored red. Wt, wild-type; Mut, mutant; NC, negative control. (D) The mRNA level of Foxp3 in CD4+ T cells transfected with miR-210 mimic and miR-210 inhibitor. (E) The protein level of Foxp3 in CD4+ T cells transfected with miR-210 mimic and miR-210 inhibitor. (F) ELISA result of IL-10 in CD4+ T cells transfected with miR-210 inhibitor and stimulated by IL-6 and PBS. (G) Western blot result of Foxp3 in CD4+ T cells transfected with miR-210 inhibitor stimulated by IL-6 and PBS. (H) Western blot result of α-SMA, collagen I and collagen III in transfected CD4+ T cells co-cultured with atrium mechanocyte. *$P < 0.05$, **$P < 0.01$ vs normal or mimic NC or inhibitor NC; #$P < 0.05$, ##$P < 0.01$ vs inhibitor NC or IL-6 + inhibitor NC or Ang II + mimic NC; &$P < 0.05$ vs Ang II + inhibitor NC.

Treg cells modified with miR-210 affect mice with AF

To corroborate the effect of Treg cells on AF *in vivo*, an animal model of atrial fibrosis was established. A miR-210 antagomir and a control were transfected into Treg cells and then injected into the animal model. The results of H&E stain-ing indicated that the transverse sections of cardiomyocytes in the model group were significantly larger than those in the control group, while the transverse sections of cardio-myocytes in the miR-210 antagomir group were markedly smaller than those in the antagomir group (Figs. 5A and 5C).
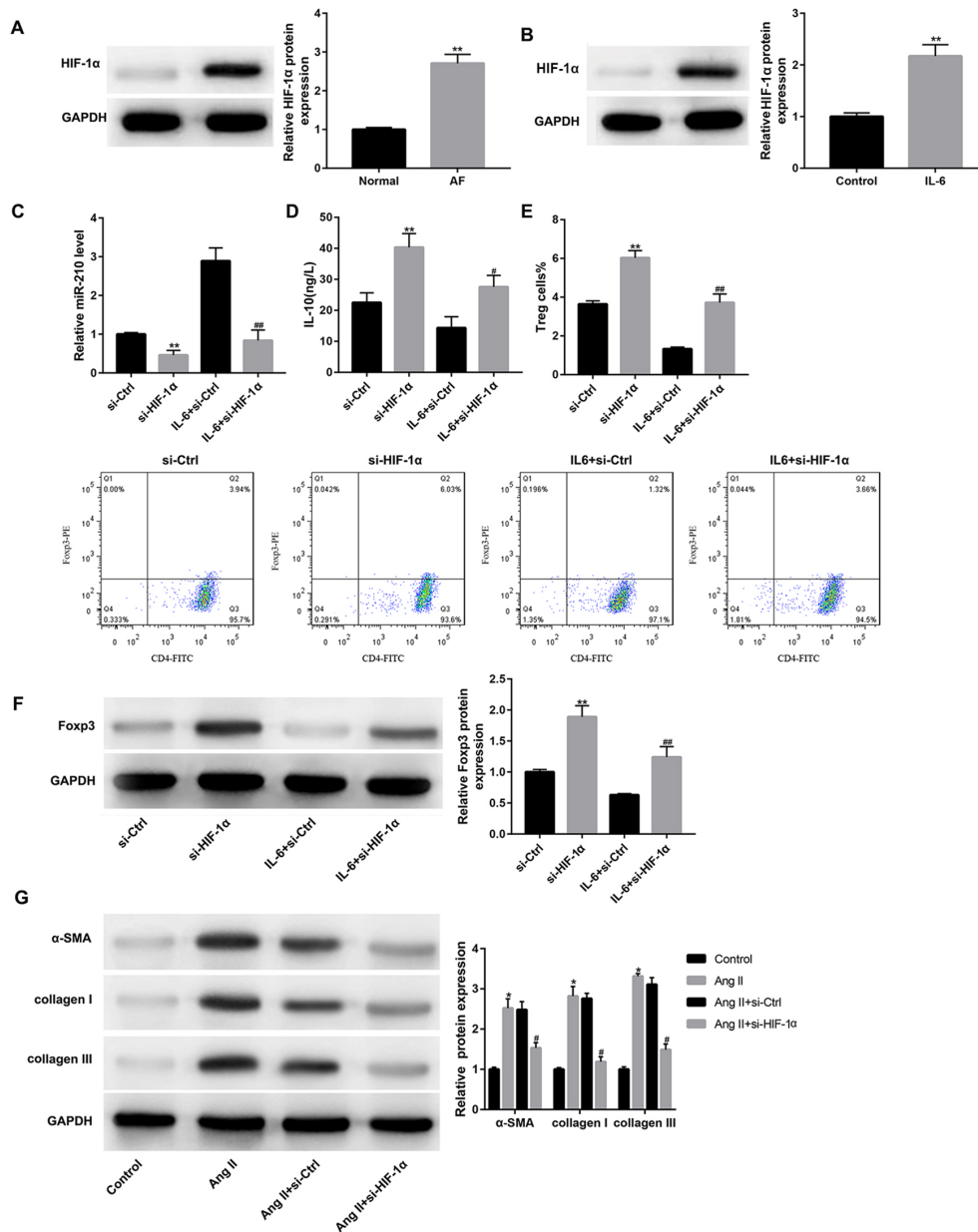
Fig. 4. IL-6 promotes expression of miR-210 via regulating HIF-1α. (A) Western blot result of HIF-1α in right atrial tissue of AF patients and normal subjects. (B) The protein level of HIF-1α in CD4+ T cells stimulated by IL-6 and PBS. (C) The mRNA level of miR-210 in CD4+ T cells transfected with HIF-1α siRNA and stimulated by IL-6 and PBS. (D) ELISA result of IL-10 in CD4+ T cells transfected with HIF-1α siRNA and stimulated by IL-6 and PBS. (E) FACS analysis of Treg cells (FoxP3) in CD4+ T cells transfected with HIF-1α siRNA and stimulated by IL-6 and PBS. (F) Western blot result of Foxp3 in CD4+ T cells in CD4+ T cells transfected with HIF-1α siRNA and stimulated by IL-6 and PBS. (G) Western blot result of α-SMA, collagen I and collagen III in transfected CD4+ T cells co-cultured with atrium mechanocyte. $*P < 0.05$, $**P < 0.01$ vs normal or control or si-Ctrl; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs IL-6 + si-Ctrl or Ang II + si-Ctrl.

The dark red myocardial tissue and blue collagen fibres were observed by Masson staining, and the results showed that myocardial filaments were loose and the nucleus was larger in the model group, which was manifested as more severe fibrosis in the model group compared to the control group. However, the miR-210 antagomir group exhibited alleviated fibrosis compared to the antagomir group (Figs. 5B and 5D).

Moreover, the enlargement of cardiac tissue resulted in an increased HW/BW ratio in the model group, but the miR-210 antagomir suppressed the increase compared to the antagomir control treatment (Fig. 5E). Finally, in comparison with the control treatment, the miR-210 antagomir markedly suppressed the expression of α-SMA, collagen I and collagen III (Fig. 5F). Overall, our data suggest that miR-210 antagomir
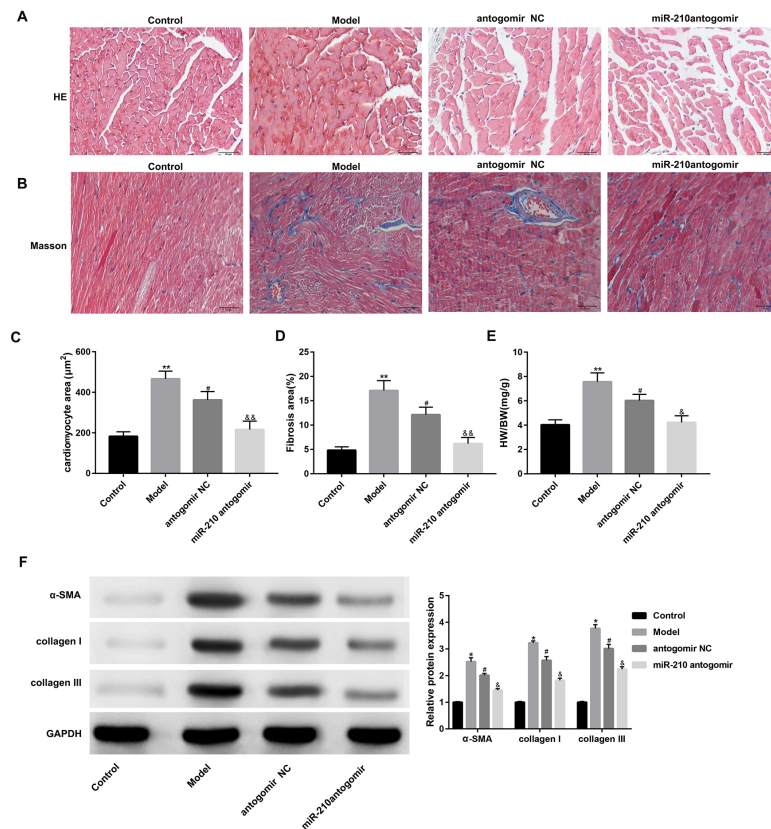
Fig. 5. Effects of Treg cells modified with miR-210 on mice with AF. Animal model of mouse atrial fibrosis was established, and miR-210 antogomir and control were transfected into Treg cells. (A) H&E staining result in each group. Scale bars = 20 μM. (B) Masson staining result in each group. Scale bars = 20 μM. (C) The cardiomyocyte area in each group. (D) The fibrosis area in each group. (E) The ratio of heart weight (HW)/body weight (BW) in each group. (F) Western blot result of α-SMA, collagen I and collagen III in each group. $*P < 0.05$, $**P < 0.01$ vs control; $^{\#}P < 0.05$ vs model; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs antogomir NC.

alleviates fibrosis in mice with AF via enhancing Treg function.

DISCUSSION

In the present study, we identified a negative correlation between IL-6 levels and IL-10 levels in patients with AF. First, we found that IL-6 suppresses Treg function and promotes mechanocyte fibrosis. At the same time, we proved that Foxp3 was a direct target gene of miR-210 with a dual-luciferase reporter assay and revealed that IL-6 inhibits Treg function by regulating the targeting of Foxp3 by miR-210. Next, to explore the mechanism by which IL-6 regulates miR-210, the expression of HIF-1α was measured by western blot, and the results suggested that IL-6 promotes expression of miR-210 via regulating HIF-1α. Finally, we validated that miR-210 inhibits Treg function and improves fibrosis in mice with AF *in vivo*.

Both the occurrence and the maintenance of AF are associated with pathophysiological changes in atria, mainly displayed as atrial remodelling, and atrial fibrosis is the main manifestation of atrial structural remodelling (Lendeckel et al., 2012). Previous studies have shown that Tregs play a critical role in the development of pulmonary fibrosis. For ex-

ample, Treg depletion decelerated the process of pulmonary fibrosis and hindered fibrocyte recruitment to the lung (Xiong et al., 2015), and Tregs attenuated cardiac hypertrophy and ventricular remodelling induced by Ang II and stress overload (Kanellakis et al., 2011). Further research on animal models of myocardial infarction found that Tregs reduce cardiac aggravated ventricular remodelling (Dobaczewski et al., 2010). Our findings suggest that the levels of IL-6 and IL-10 (Tregs) were negatively correlated; moreover, IL-6 suppressed Treg cell function and promoted fibrosis of mechanocytes, which are consistent with the above findings.

MiR-210 is a hypoxia-induced miRNA that plays important roles in many physiological and pathological processes in humans, including the fibrosis of various organs. MiRNAs were validated as useful diagnostic biomarkers in patients with liver disease with cystic fibrosis (Cook et al., 2015). Bodempudi et al. (2014) found that miR-210 expression markedly increased in fibroblasts of idiopathic pulmonary fibrosis in response to hypoxia and that knockdown of miR-210 reduced hypoxia-induced fibroblast proliferation. MiRNAs have been shown to play an important role in immune responses, including the phenotypic stability of Treg cells (O'Connell et al., 2010). The expression of FOXP3 is required for Treg development

and appears to facilitate the differentiation of Treg cells via genetic programming (Marson et al., 2007). A growing body of research has revealed that miRNAs regulate FOXP3 expression. Fayyad-Kazan et al. (2012) revealed that miR-24 negatively regulated FOXP3 expression by directly binding to its target sites in its 3'-UTR, while miR-95, which is highly expressed in adult peripheral blood Tregs, positively regulated FOXP3 expression. Becker et al. (2018) reported that miR-466a targeting of TGF-β2 contributes to FoxP3+ regulatory T cell differentiation. In this study, treatment with a miR-210 inhibitor significantly promoted the expression of IL-10 (Tregs) and Foxp3 in CD4+ T cells compared to control treatments (Figs. 3F and 3G), indicating that miR-210 regulated the function of Treg cells by targeting FOXP3, which is consistent with previous data (Zhao et al., 2014). Interestingly, IL-6 was demonstrated to be a modulator of miR-210 in this pathway.

HIF-1α is a transcription factor that consistently upregulates miR-210 (Kelly et al., 2011; Nakada et al., 2011). Studies have reported that IL-6 promotes the expression of miR-17 by regulating HIF-1α (Yang et al., 2016); another study showed that hypoxia leads to simultaneous increased expression of IL-6 and miR-210 in human pancreatic cancer cells (Bao et al., 2012). In this study, we observed increases in HIF-1α expression in CD4+ T cells stimulated by IL-6 and decreases in miR-210 expression in CD4+ T cells treated with IL-6+HIF-1α siRNA, further demonstrating that IL-6 promotes the expression of miR-210 via regulating HIF-1α. Duan et al. (2016) revealed that miR-210 regulates IL-6 by targeting NR1D2 in patients with cryptorchidism. NR1D2 (nuclear receptor family 1 member 2), also called REV-ERBβ, is very similar to NR1D1. REV-ERBα (NR1D1) activation prevents the development of cardiac hypertrophy, reduces fibrosis, and halts the progression of advanced heart failure in mouse models (Zhang et al., 2017). Therefore, there might be a feed-back loop that regulates the levels of IL-6 and miR-210, further modulating atrial fibrosis.

There are several potential limitations in this study. First, the sample size is small. Second, different types of AF, paroxysmal, persistent and permanent, were not considered when we collected data from patients with AF. Third, we did not perform animal experiments *in vivo* on AF promoting the expression of IL-6 by suppressing HIF-1a/miR-210/Foxp3 in Tregs, which would have better validated the role of IL-6 in regulating Treg function.

In conclusion, our data demonstrate for the first time a new role for IL-6-miR-210 in the regulation of Treg function in atrial fibrosis. This study provides an important clue to help elucidate the pathogenesis of AF and offers novel avenues to control AF.

AUTHOR CONTRIBUTIONS

Y.W.C. and G.D.C. conceived and designed the study. Y.W.C., G.D.C., X.J.C., and Y.P.L. performed the experiments. X.J.C. and H.Y.L. wrote the paper. Y.W.C., Y.P.L., D.C., Y.T., and H.Q.S. reviewed and edited the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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