Integrated analysis of microRNA and mRNA expression profiles in the left atrium of patients with nonvalvular paroxysmal atrial fibrillation: Role of miR-146b-5p in atrial fibrosis

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BACKGROUND Studies have reported that the integrated analysis of microRNA (miRNA)-messenger RNA (mRNA) expression is valuable in exploring gene regulation systemically.

OBJECTIVES The objectives of this study were to identify miRNAs and genes involved in atrial fibrillation and to explore the mechanisms underlying atrial fibrosis.

METHODS We used microarrays to compare the differences in both miRNA and mRNA expression profiles in the left atrial appendage of patients with nonvalvular paroxysmal atrial fibrillation and healthy controls. Furthermore, the quantitative real-time polymerase chain reaction was used to confirm the reliability of the microarray data, prediction of the adopted databases, and Ingenuity Pathway Analysis of miRNA-mRNA expression in order to identify the miRNA target genes, examine the functions and pathways in which the target genes are involved, and construct an miRNA-target gene regulatory network. We further investigated the roles of miRNA-146b-5p in the mechanisms of atrial fibrosis.

RESULTS We identified 10 differentially expressed miRNAs and 624 differentially expressed mRNAs, among which only 1 miRNA-target gene pair miR-146b-5p and tissue inhibitor of metalloproteinase 4 (TIMP-4) were constructed. The validated results revealed that miR-146b-5p, matrix metalloproteinase 9, and collagen content were upregulated whereas TIMP-4 was downregulated in patients with atrial fibrillation. After the transfection of miR-146b-5p into cardiac fibroblasts, TIMP-4 expression was markedly reduced and collagen content was increased. Moreover, luciferase results confirmed that TIMP-4 was a target of miR-146b-5p.

CONCLUSION The identified miRNA and mRNA may represent a potentially novel molecular regulatory network, which may provide a better understanding of the molecular basis of remodeling in atrial fibrillation. miR-146b-5p probably acts as an intracellular mediator in the maladaptive remodeling in atrial fibrosis in atrial fibrillation.

KEYWORDS MicroRNA; Atrial fibrillation; Nonvalvular; Fibrosis; MicroRNA-146

ABBREVIATIONS AF = atrial fibrillation; HC = healthy control; IPA = Ingenuity Pathway Analysis; LAA = left atrial appendage; MMP = matrix metalloproteinase; mRNA = messenger RNA; miRNA = microRNA; PAF = paroxysmal atrial fibrillation; qPCR = quantitative polymerase chain reaction; RT-PCR = real-time polymerase chain reaction; TIMP = tissue inhibitor of metalloproteinase

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Introduction

Atrial fibrillation (AF) is the most common sustained cardiac rhythm disorder, and its prevalence has been increasing. The fundamental mechanisms underlying AF have long been debated, and electrical, contractile, and structural remodeling...
are each important contributors to the AF substrate. Atrial fibrosis has been considered a key element of AF. Increased collagen deposition has been documented in patients with AF and in patients with valvular AF. Although these findings highlight the association between atrial fibrosis and AF, only a few causal studies have reported the regulatory mechanism of atrial fibrosis in nonvalvular paroxysmal atrial fibrillation (PAF), which is a major health issue in the world.

MicroRNAs (miRNAs) are endogenous ~23-nucleotide RNAs that play important gene-regulating roles in animals and plants by pairing with messenger RNAs (mRNAs) of protein-coding genes to direct their posttranscriptional regulation.

Therefore, measuring miRNA expression can be useful for gene regulation studies at the system level, especially when mRNA measurements are combined with miRNA profiling data. Some investigators have explored the association of miRNAs and gene expression profiles and reported that miRNAs have evolved to regulate gene expression programs.

Although Dawson, Cooley, and coworkers showed that miR-29 and miR-21 are likely to play a role in atrial fibrotic remodeling caused by congestive heart failure and valvular heart disease, no study has focused on the impact of miRNAs on the mRNA expression levels in the remodeling of atrial fibrosis in nonvalvular PAF. To shed light on the interplay between mRNAs and miRNAs in nonvalvular PAF and verify that altered expression of miRNAs can modulate the levels of the corresponding target mRNAs, we obtained miRNA profiles in the left atrial appendage (LAA). We then analyzed these miRNA expression profiles with the gene expression profiles of the same populations and identified the potential regulatory mechanisms governing atrial fibrosis.

Methods
A detailed description of the methods is available in Online Supplemental Methods.

Study population
The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institutional human research committee. It was approved by the Ethics Committee for the Use of Human Samples of Capital Medical University. Thirty patients with PAF were enrolled in this study at Beijing Anzhen Hospital according to the guidelines of the American Heart Association/American College of Cardiology. Informed consent was obtained from all subjects. All patients underwent Dallas lesion set and exclusion of the LAA using a video-assisted surgical approach. Seventeen LAA specimens obtained from healthy donor hearts without any evidence of AF or cardiovascular diseases during heart transplantation served as healthy controls (HCs). These tissues are considered as surgical waste in accordance with Chinese ethical laws. The tissue sample from each LAA was divided into 2 groups for pathological and molecular biological experiments.

Total RNA (including miRNA) isolation
The total RNA containing small RNAs was extracted from LAA samples using the TRIzol reagent (Invitrogen) and purified with the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer’s protocol.

mRNA expression assays and analysis
We used the 44K human whole genome oligonucleotide microarray (Agilent Technologies, Santa Clara, CA), and transcription was carried out using a low RNA input fluorescent linear amplification kit (Agilent Technologies, Santa Clara, CA) in the presence of Cyanine 3-CTP (Cy3-CTP) and Cyanine 5-CTP (Cy5-CTP). The data were recorded and transferred to text files using the Feature Extraction 9.3 (Agilent Technologies, Santa Clara, CA) and GeneSpring 12.0 (Agilent Technologies) software. A fold change > 2 or < 0.5 was used for analysis.

miRNA reverse transcription and TaqMan low-density array quantitative polymerase chain reaction
To assess the levels of specific miRNAs in the LAA samples, the reverse transcription reaction was carried out according to the manufacturer’s instructions. The real-time polymerase chain reaction (RT-PCR) was carried out as follows: 16°C for 2 minutes, 42°C for 1 minute, and 40 cycles of 50°C for 1 second, followed by incubation at 85°C for 5 minutes using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). After the RT-PCR was completed, the miRNA expression profiles were acquired using the TaqMan low-density miRNA array according to the manufacturer’s instructions. The quantitative PCR (qPCR) was carried out using a 7900HT thermocycler (Applied Biosystems) under the following recommended conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Raw Cq values were calculated using the SDS software version 2.3 (Applied Biosystems).

Statistical analysis of the miRNA data
Statistical analysis of the microarrays (SAM, version 3.02) was performed to determine the significance of differentially expressed miRNAs. To select the differentially expressed miRNAs, we used threshold values of ≥2-fold changes and a false discovery rate correction for multiple comparisons of < .05 to examine the differentially expressed miRNAs compared with HCs.

Ingenuity Pathway Analysis
The selected miRNAs were further analyzed to identify the networks and pathways. For this purpose, we used the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) software. This software identifies the putative targets for the input miRNA(s), integrates them with the mRNA microarray profile data, and then develops networks and functions of the genes/targets.
Quantitative RT-PCR
To obtain miRNAs, reverse transcription of RNA was conducted using the TaqMan microRNA Reverse Transcription Kit (ABI) (Applied Biosystems) according to the manufacturer’s instructions. To obtain miRNAs, the total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen) and random primers. The PCR was conducted using a 7900HT Sequence Detection System (Applied Biosystems).

Western blotting analysis
Western blotting analysis was performed to determine matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase (TIMP), and collagen expression. The reactions were developed with enhanced chemiluminescence reagents (NEN Life Science Products, Boston, MA, or Pierce Chemical Co, Rockford, IL), and the images were obtained by exposure to x-ray films. The films were digitized and quantified with the ImageQuant TL software (GE-Healthcare, Piscataway, NJ). All antibodies were purchased from Merck Millipore, Billerica, MA.

Plasmid construction
The pmirGLO Dual-Luciferase miRNA Target Expression Vector (7350 bp; Promega Corporation, Madison, WI) was used to confirm the function of the putative miR-146b-5p-binding site in the 3’ untranslated regions (3’-UTR) of TIMP-4. In contrast, site-directed mutagenesis against the putative miR-146b-5p-binding site located in the 3’-UTR of TIMP-4 was induced by the standard PCR to generate the TIMP-4-mut-3’-UTR reporter plasmid.

Dual luciferase reporter gene assay
NIH/3T3 cells were divided into groups according to the concentration of the transfection plasmid. NIH/3T3 cells were seeded in triplicate in 96-well plates, allowed to settle for 24 hours, and then cotransfected with different plasmid concentrations by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase and Renilla activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay Kit (Promega Corporation, Madison, WI) according to the manufacturer’s instructions.

Cell culture and transfection
Primary cardiac fibroblasts were isolated from 1- to 3-day-old C57BL6 mice by using 0.25% trypsin. The fibroblasts were cultured in Claycomb media supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 0.1 mol/L of norepinephrine (Sigma), 2 mmol/L of L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen) in a humidified 5% CO2 incubator at 37°C. The fibroblasts were maintained at subconfluent densities and used when grown to passages 2 or 3.

For transfection, cells were washed with serum-free medium once and then incubated with 4 mL of serum-free medium for 4–6 hours. miRNA (or miRNA inhibitor and scrambled miRNA) and Lipofectamine 2000 were separately mixed with 500 μL of Opti-MEM I Reduced Serum Medium (Gibco Life Technologies, Grand Island, NY) for 5 minutes. Then, the 2 mixtures were combined and incubated for 20 minutes at room temperature. The Lipofectamine 2000-miRNA mixture was added to the cells and incubated at 37°C for 6 hours. Subsequently, 5 mL of fresh medium containing 10% fetal bovine serum was added to the flasks and the cells were maintained in the culture until the experiments were conducted.

Statistical analysis
All results for continuous variables are expressed as mean ± SD, and categorical variables are expressed as the number of cases and percentage. The significance of the differences between the groups was assessed by using the Student t test or 1-way analysis of variance for continuous variables and the χ2 test for categorical variables. Logistic regression analyses were performed to identify the relationship between the variables. All tests were 2-tailed, and a P value of <.05 was considered to indicate significance. The data were analyzed with SPSS for Windows version 12.0 (SPSS Inc, Chicago, IL).

Results
A total of 47 subjects were studied. LAA samples were obtained from 30 patients with PAF. The time since the first diagnosis of AF was ~2 years. The mean left atrial diameter was 53.1 ± 2.2 mm. The HC group consisted of 17 LAA samples obtained from transplant donors after heart transplantation. The clinical characteristics of the 2 study populations are summarized in Table 1.

mRNA profiles of patients with AF vs HCs
To determine the levels of mRNA and miRNAs in the LAA in the patients, we performed mRNA and miRNA profiling in 8 patients with AF and in 8 HCs.

Table 1  Characteristics of the study cohort

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HCs (n = 17)</th>
<th>Patients with PAF (n = 30)</th>
<th>P</th>
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<tr>
<td>Demographic characteristics</td>
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<tr>
<td>Sex: male</td>
<td>13(76%)</td>
<td>19(63%)</td>
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<td>Age (y)</td>
<td>43±1.3</td>
<td>47.3±6.8</td>
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<td>Functional status</td>
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<tr>
<td>LVEF (%)</td>
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<td>62.5±7.4</td>
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<td>2.3±0.2</td>
<td>.203</td>
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<td>AF duration (y)</td>
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<td>.001</td>
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<td>LAD (mm)</td>
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<td>53.1±2.2</td>
<td>.003</td>
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<tr>
<td>LVEDD (mm)</td>
<td>42.5±9.1</td>
<td>45.1±7.3</td>
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<td>β-Blocker</td>
<td>0</td>
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<td>Amiodarone</td>
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<td>5(17%)</td>
<td>.143</td>
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<tr>
<td>Digitalis</td>
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<td>2(7%)</td>
<td>.528</td>
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<tr>
<td>Aspirin/clopidogrel/warfarin</td>
<td>0</td>
<td>4(13%)</td>
<td>.281</td>
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</table>

Data are presented as mean ± SD or as number.
AF = atrial fibrillation; HC = healthy control; LAD = left atrial dimension; LVEDD = left ventricular end-diastolic diameter; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; PAF = paroxysmal atrial fibrillation.

*Statistically significant, PAF group vs HC group.
The gene expression profiles were assayed using an mRNA microarray, and we observed that the expression of 422 genes was upregulated and the expression of 202 genes was downregulated in patients with PAF, with more than a 2-fold change compared to HCs ($P < .05$; Online Supplemental Table 1).

**Prediction of biological function and AF associated with the mRNA expression profile**

The mRNA expression profiles were analyzed using the IPA bioinformatics tool to predict the biological changes associated with AF. We found that 95–225 genes were associated with diseases such as connective tissue disorders, immunological disorders, inflammatory response, skeletal and muscular disorders, and cancer (Figure 1). In addition, predictive analysis of the AF-related genes showed that 12 genes were involved in AF: TIMP-4, MMP-9, F2, KCNMB2, KCNE1, SCN18, KCNA4, SCN4A, SLC8A1, IL-6, PTGS2, and PTGS1.

**miRNA profiles of patients with AF vs HCs**

The miRNA expression profiles of patients with AF and HCs were compared. The analysis of variance revealed that 10 miRNAs were differentially expressed between patients with AF and HCs (>2-fold change; $P < .05$), as illustrated in the volcano plot shown in Figure 2A. These miRNAs were miR-146b-5p, miR-155, miR-19b, miR-142-3p, miR-486-5p, miR-223, miR-193b, miR-519b-3p, miR-301b, and miR-193a-5p.

RT-PCR analysis confirmed the significant upregulation of miRNAs miR-146b-5p, miR-155, miR-19b, miR-142-3p, miR-486-5p, miR-223, miR-193b, miR-519b-3p, and miR-301b as well as the significant downregulation of miR-193a-5p (Figure 2B). In particular, miR-146b-5p, miR-155, and miR-19b showed the most pronounced change among the 10 differentially expressed miRNAs: the expression was elevated by 4 times in patients with AF compared with HCs.

To confirm the findings of the miRNA profile analysis, we determined the expression of the selected miRNAs in the entire cohort (n = 47) by using TaqMan qPCR. As shown in Online Supplemental Figure 1, the expression of miR-146b-5p was significantly increased in patients with PAF.

**Integrated analysis of dysregulated miRNAs and mRNAs**

miRNAs modulate gene expression through both mRNA degradation and translational repression mechanisms, and the miRNA-mRNA regulatory networks are highly complex.
IPA predicted the biological processes of AF that were most affected based on both the miRNAs and their targets and the mRNA profiling results. Interestingly, only 1 gene, TIMP-4, from our mRNA microarray data was found to be a target of miR-146b-5p among the 10 differentially expressed miRNAs, as miR-146b-5p showed the most pronounced changes.

Association of TIMP-4/MMP-9 with atrial fibrosis in AF

TIMP-4 belongs to the TIMP gene family. The proteins encoded by this gene family are inhibitors of MMPs, a group of peptidases involved in the degradation of the extracellular matrix. Matrix synthesis and degradation are mainly regulated by the balance between the MMP and the TIMP axis. In the heart, TIMP-4 and MMP-9 are predominantly involved in cardiac remodeling, which may cause atrial fibrosis in AF. MMP-9 remains latent but is activated in certain pathological conditions and degrades the matrix, resulting in the deposition of oxidized collagen and leading to fibrosis. The histopathological findings of the LAA in patients with PAF were characterized by a diffuse increase in interstitial fibrosis with areas of focal fibrosis (Figure 3A).

To determine the relation between TIMP-4 and MMP-9, we measured the expression of TIMP-4 and MMP-9 in the entire cohort (n = 47). TaqMan qPCR showed that the relative expression level of TIMP-4 was significantly lower in the AF group than in the HC group. Conversely, MMP-9 showed significantly higher expression in the AF group than in the HC group. Moreover, the ratio of MMP-9 to TIMP-4 was significantly higher in the AF group than in the HC group (Figure 3B). Interestingly, significant negative correlations were found between the mRNA expression level of TIMP-4 and MMP-9 in the AF group (Figure 3C). Western blotting analysis showed that TIMP-4 protein expression was significantly lower in the AF group than in the HC group (Figure 3D). Moreover, the expression of MMP-9 and collagen type I was significantly higher in the AF group than in the HC group (Figures 3E and 3F).

Role of miR-146b-5p and its target

TIMP-4 in atrial fibrosis

The association of TIMP-4 with miR-146b-5p indicated their role in atria fibrosis. We further found that the 3-UTR region of TIMP-4 region has only 1 highly conserved miR-146b-5p-binding site (Figure 4A). After NIH/3T3 cells were cotransfected with reconstructive vectors and the normalizing vector pRL-TK containing Renilla luciferase, the luciferase activity of pGL4-TIMP-4 was dramatically inhibited by overexpression of miR-146b-5p on the cotransfection of NIH/3T3 cells with miR-146b-5p (10 nmol/L). This downregulation was efficiently prevented by the miR-146b-5p inhibitor. In contrast, mutant reporters were not repressed by miR-146b-5p, confirming that the target site directly mediates repression of luciferase activity through seed-specific binding (Figure 4B).

The ability of miR-146b-5p to repress the expression of TIMP-4 was further verified by Western blotting analysis in mouse cardiac fibroblasts transfected with miR-146b-5p. This downregulation was efficiently prevented by the miR-146b-5p inhibitor. Moreover, MMP-9 and collagen type I expression was significantly increased, which was in contrast to the expression pattern of TIMP-4 (Figure 4C). Taken together, these results demonstrate that miR-146b-5p might contribute to atrial fibrosis via TIMP-4.

Discussion

To our knowledge, this is the first study of integrated target prediction with miRNA and mRNA expression data; here, we have identified the involved miRNAs and their associated targets in the left atrium in patients with nonvalvular PAF. The results showed that the expression of miR-146b-5p,
**Figure 4** Confirmation of TIMP-4 as the target gene of miR-146-5p. (A) The TIMP-4 3’-UTR region has only 1 highly conserved miR-146b-5p-binding site. (B) miR-146b-5p significantly reduced the luciferase activities of the pGL4-TIMP-4 reporter compared with the scrambled miRNA negative control (NC). This downregulation was efficiently prevented by the miR-146b-5p inhibitor. However, mutant reporters (TIMP-4-mut) with noncomplementary seed-binding sites were not repressed by miR-146b-5p. The blank vector (pGL4-Control) has no seed-binding site and therefore luciferase activity was not affected by miR-146b-5p. (C) Western blot for the verification of posttranscriptional repression of TIMP-4, MMP-9, and collagen type I. The protein samples were isolated from cardiac fibroblasts 48 hours after transfection with miR-146b-5p alone, the miR-146b-5p inhibitor, or a scrambled miRNA for the NC. *P < .05; **P < .001 vs HC or NC group. HC = healthy control; miRNA = microRNA; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase.
miR-155, and miR-19b was significantly increased in patients with nonvalvular PAF compared with HCs. The expression of these miRNAs and the global profiles of mRNAs were integrated, and the findings showed that only the TIMP-4 gene from our mRNA microarray data showed a high level of association with miR-146b-5p, which might contribute to PAF by promoting fibrosis, an established AF substrate. The results revealed that the upregulation of miR-146b-5p leads to the downregulation of TIMP-4, which in turn can activate MMP-9, resulting in increased collagen synthesis, which enhances atrial fibrosis.

**Comorbidities affecting miRNA expression**

Previous studies have reported that the expression of miRNAs such as miR-1, miR-328, miR-106b-25, and miR-499 are altered in valvular AF, but we did not observe any significant changes in the expression of these miRNAs in our study. This difference in the findings compared with other studies may be attributed to differences in the tissues that were sampled (LAA in the present study vs left or right atrial tissue in other studies) and the heterogeneity of human myocardial samples. AF has a complex electrical phenotype involving complex factors beyond the electrophysiological factors, and it can occur in a variety of pathological settings in 2 atria. The different types of AF have distinct underlying mechanisms, and different miRNAs may be involved in these different types of AF.

The present study showed a specific set of miRNAs in patients with nonvalvular PAF that demonstrated unique expression of miRNAs such as miR-146b-5p, miR-155, and miR-19b, which were significantly upregulated in the study subjects.

**miR-146 and TIMP-4**

miR-146 is thought to be a mediator of inflammation along with miR-155. The expression of miR-146 is upregulated by inflammatory factors such as interleukin 1 and tumor necrosis factor. miR-146 regulates a number of targets, which are mostly involved in the Toll-like receptor pathways that induce a cytokine response as part of the innate immune system. Recently, miR-146b-5p was reported to be upregulated in patients with AF, which is consistent with our findings. The potential targets of miR-146b-5p include TIMP-4, MMP16, and TGIF1 (TGFβ-induced factor homeobox 1), which are reported to be regulated in cardiomyocyte fibrosis; these targets might contribute to AF by promoting fibrosis, an established AF substrate. In this study, we found an inverse relationship between the expression of miR-146b-5p and TIMP-4 in the LAA in patients with PAF. Furthermore, the luciferase activity of putative wild-type TIMP-4 was downregulated by miR-146b-5p, but miR-146b-5p did not affect the expression of mutant target sequences. This finding suggests that TIMP-4 is a target of miR-146b-5p and that the inhibition was seed sequence specific.

**TIMP-4/MMP-9 contribute to atrial fibrosis in the remodeling of AF**

PAF is associated with structural remodeling in cardiac fibroblasts and the surrounding matrix. Matrix synthesis and degradation is mainly regulated by the balance between matrix MMPs and tissue inhibitors of the TIMP axis. In the heart, MMP-9 and TIMP-4 are predominantly involved in cardiac remodeling. MMP-9 remains latent but is activated in certain pathological conditions and degrades the matrix, resulting in the deposition of oxidized collagen and leading to fibrosis; TIMP-4 mitigates the effect of MMP-9. We selected TIMP-4 and MMP-9 to investigate the fibrosis mechanism in atrial remodeling by PAF in the present study. The results indicated that the expression of TIMP-4 was significantly lower in the LAA of patients with PAF than in the LAA of HCs at the protein level as well as the mRNA level; however, the expression of MMP-9 and collagen type I was significantly higher in patients with AF than in HCs. Moreover, a significant negative correlation was observed between the expression of TIMP-4 and MMP-9 in patients with PAF. Based on these data, it was determined that the balance between TIMP-4 and MMP-9 is associated with atrial structural remodeling in PAF.

The development of atrial fibrosis is accompanied by increased collagen content in fibroblasts. Therefore, transfection of miR-146b-5p into fibroblasts was used to investigate whether miR-146b-5p contributes to the increased collagen content via its target TIMP-4. Western blotting analysis showed that the overexpression of miR-146b-5p could downregulate the expression of TIMP-4 in cardiac fibroblasts. Moreover, downregulation of TIMP-4 could induce upregulation of MMP-9. Upregulation of MMP-9 could markedly increase the level of collagen content. Moreover, the miR-146b-5p inhibitor could increase TIMP-4 expression and also abolish the increase in MMP-9 expression and collagen content induced by miR-146b-5p in cardiac fibroblasts. Together, these results suggest that miR-146b-5p activates atrial fibrosis by disturbing the balance between the MMP and TIMP axis and finally increases the level of collagen content in cardiac fibroblasts.

**Study limitations**

It is unlikely that a single miRNA is the sole contributor to atrial fibrosis in complex conditions such as AF. Indeed, the expression of a substantial number of miRNAs is altered in AF and may be pathophysiologically important. Moreover, the levels of miRNAs may be affected by multiple factors such as changes in expression in the tissue, the release of miRNAs by the cells, and the stability of miRNAs. If a miRNA is hypothesized to be directly involved in a cardiomyocyte response, the relevant expression changes must be assessed in cardiomyocytes; If a miRNA is hypothesized to be directly involved in a cardiomyocyte response the relevant expression-changes must be assessed in cardiomyocytes. If it is hypothesized to be directly involved in a fibroblast response (e.g. ECM regulation,
fibrosis) the expression must be verified in fibroblasts, because changes in overall tissue-expression do not necessarily reflect alterations in specific cell-types. Further study need to verify these changes in miRNA expression in different heart cell compartments. Furthermore, it should be noted that although our study identified miR-146b-5p as an intracellular mediator with an active role in the maladaptive remodeling of atrial fibrosis in patients with non-valvular PAF, our study did not provide evidence for this in experimental AF models. A better understanding of the biological significance of these changes in miRNA expression in patients with PAF would be possible with the development of experimental models in which the levels of 1 or more miRNAs could be precisely manipulated.

Conclusion
We identified 10 differentially expressed miRNAs and 624 differentially expressed mRNAs, but constructed only 1 posttranscriptional regulatory network miRNA-target gene pair: miR-146b-5p and TIMP-4 in the LAA of patients with nonvalvular AF. The correlation between the expression levels of miR-146b-5p, the balance between TIMP-4 and MMP-9, and the level of collagen content support the hypothesis that miR-146b-5p acts as an intracellular mediator with an active role in the maladaptive remodeling of atrial fibrosis in patients with PAF.

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Appendix
Supplementary Materials
Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.hrthm.2015.01.026.

References
CLINICAL PERSPECTIVES

According to the World Health Organization, 1 in every 200 individuals has nonvalvular atrial fibrillation (AF). Despite advances in this field, the traditional pharmacological approaches have proved to be weak. Structural remodeling has been observed in both clinical and experimental AF and is an important feature of the AF substrate, as it results in fibrosis that alters atrial tissue composition and function. MicroRNAs (miRNAs) are small RNA sequences (18–22 nucleotides) that regulate protein transcription and messenger RNA stability. The present study, which is an integrative study of the global profiles of miRNAs and messenger RNAs, showed that miR-146b-5p is a potential contributor to atrial fibrosis in nonvalvular PAF. We found that miR-146b-5p is upregulated in patients with nonvalvular AF compared with HCs, followed by downregulation of its target tissue inhibitor of metalloproteinase 4; moreover, luciferase activity confirmed that it is the target of miR-146b-5p. Furthermore, in vitro overexpression of miR-146b-5p in cardiac fibroblasts decreased the expression of tissue inhibitor of metalloproteinase 4, which increased the expression of matrix metallopeptidase 9 and collagen type I, whereas inhibition of miR-146b-5p suppressed collagen expression. Thus, our results clearly show that miR-146b-5p has a role in fibrotic AF-maintaining substrate via promotion of collagen synthesis. Based on this, it is could be speculated that the induction of miR-146b-5p in the fibroblasts of diseased hearts might contribute, at least partially, to the increase in atrial fibrosis in AF. If this speculation is true, miR-146b-5p could be a potential therapeutic target for AF prevention or therapy for atrial fibrosis.

AUTHORS

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