MicroRNA29
A Mechanistic Contributor and Potential Biomarker in Atrial Fibrillation

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Background—Congestive heart failure (CHF) causes atrial fibrotic remodeling, a substrate for atrial fibrillation (AF) maintenance. MicroRNA29 (miR29) targets extracellular matrix proteins. In the present study, we examined miR29b changes in patients with AF and/or CHF and in a CHF-related AF animal model and assessed its potential role in controlling atrial fibrous tissue production.

Methods and Results—Control dogs were compared with dogs subjected to ventricular tachypacing for 24 hours, 1 week, or 2 weeks to induce CHF. Atrial miR29b expression decreased within 24 hours in both whole atrial tissue and atrial fibroblasts (~87% and ~92% versus control, respectively; p<0.001 for both) and remained decreased throughout the time course. Expression of miR29b extracellular matrix target genes collagen-1A1 (COL1A1), collagen-3A1 (COL3A1), and fibrillin increased significantly in CHF fibroblasts. Lentivirus-mediated miR29b knockdown in canine atrial fibroblasts (~68%; p<0.01) enhanced COL1A1, COL3A1, and fibrillin mRNA expression by 28% (p<0.01), 19% (p<0.05), and 20% (p<0.05), respectively, versus empty virus–infected fibroblasts and increased COL1A1 protein expression by 90% (p<0.05). In contrast, 3-fold overexpression of miR29b decreased COL1A1, COL3A1, and fibrillin mRNA by 65%, 62%, and 61% (all p<0.001), respectively, versus scrambled control and decreased COL1A1 protein by 60% (p<0.05). MiR29b plasma levels were decreased in patients with CHF or AF (by 53% and 54%, respectively; both p<0.001) and were further decreased in patients with both AF and CHF (by 84%; p<0.001). MiR29b expression was also reduced in the atria of chronic AF patients (by 54% versus sinus rhythm; p<0.05). Adeno-associated viral–mediated knockdown of miR29b in mice significantly increased atrial COL1A1 mRNA expression and cardiac tissue collagen content.

Conclusions—MiR29 likely plays a role in atrial fibrotic remodeling and may have value as a biomarker and/or therapeutic target. (Circulation. 2013;127:1466-1475.)

Key Words: arrhythmia ■ biomarkers ■ congestive heart failure ■ fibrosis ■ microRNA

Cardiac structural remodeling, particularly atrial fibrosis, plays a major role in atrial fibrillation (AF). Congestive heart failure (CHF) promotes atrial structural remodeling. The fibrotic response is complex and incompletely understood. MicroRNAs (miRs) are evolutionarily conserved short, noncoding RNAs that block mRNA translation and/or promote mRNA breakdown of target genes. Recently, miR21, miR29b, and miR133a were shown to be potentially involved in cardiac fibrotic responses. MiR21 enhances extracellular signal-related kinase/mitogen-activated protein kinase activity and promotes fibroblast survival; miR21 knockdown in rats subjected to myocardial infarction (MI) decreases AF duration and atrial fibrosis. MiR29b is decreased in the MI border zone, leading to derepression of extracellular matrix (ECM) genes and ECM deposition. MiR133a is decreased in left ventricular hypertrophy and directly targets connective tissue growth factor, which promotes fibrosis.

Clinical Perspective on p 1475

We designed this study to evaluate the potential importance of miR29b in AF. We first assessed miR29b expression in atrial tissues from an animal AF model with prominent fibrotic changes. We then proceeded to manipulate miR29b in vitro using lentiviruses and saw changes in corresponding miR29b ECM target genes at the mRNA and protein levels. Because plasma miR concentrations change in patients with MI, coronary artery
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disease, and heart failure and miRs may represent useful biomarkers,10–12 we measured plasma miR21, miR29b, miR133a, and miR15 concentrations in patients with AF and/or CHF. We then quantified miR29b and miR133a expression in atrial tissue from AF patients. Finally, we used adenoassociated viruses to infect mice with miR29b knockdown or “sponge” viruses to determine the manner in which reduced miR29b expression affects tissue collagen gene expression. Our results support a potential role for miR29b in AF.

Methods
A detailed description of Methods is available in the online-only Data Supplement.

Patient Characteristics
Plasma samples were obtained from 33 patients (Table 1) with persistent AF according to American Heart Association/American College of Cardiology guidelines.13 AF symptom score was assessed by European Society of Cardiology guidelines.14 Patients had to be clinically compensated with resting heart rate 50 to 100 bpm during AF at the time of blood sampling. Thirty-two patients with CHF and no history of AF (as ascertained by ECG on at least 3 clinic visits) constituted the CHF-only population. The diagnosis of CHF was based on the American College of Cardiology/American Heart Association 2005 guidelines,13 with typical symptoms and reduced echocardiographic left ventricular ejection fraction. New York Heart Association class IV patients were excluded. The AF group was subdivided into patients with (n=16) and without (n=17) CHF on the basis of the same clinical criteria. Controls were patients from surgical outpatient clinics (n=30) in whom heart disease (ECG and clinical examination), chronic inflammatory diseases, history of cancer, liver disease, or neurodegenerative disease were excluded. We measured miRs in plasma according to Mitchell et al.16 Written informed consent under institutional review board–approved protocols (Klinikum Grosshadern, University of Munich) was obtained in all cases.

Right atrial appendages were dissected from a separate population (Table 2) including 19 patients with sinus rhythm, 9 with paroxysmal AF, and 8 with chronic AF (duration >6 months). Experimental protocols were approved by the ethics committee of the Medical Faculty Mannheim, Heidelberg University (No. 2011-216 N-MA). Each patient gave written informed consent. After excision, right atrial appendages were snap-frozen in liquid nitrogen.

Canine Model
Animal handling procedures followed National Institutes of Health guidelines (http://oacu.od.nih.gov/training/index.htm) and were approved by the Animals Research Ethics Committee of the Montreal Heart Institute. Ninety-four mongrel dogs (weight, 22–36 kg) were divided into 5 groups: control (n=32), sham (n=5), 24-hour ventricular tachypacing (VTP) (n=19), 1-week VTP (n=16), and 2-week VTP (n=22). Sham and VTP dogs were chronically instrumented as

Table 1.  Patient Characteristics: Plasma Samples

<table>
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<tr>
<th></th>
<th>Controls</th>
<th>AF, No CHF</th>
<th>No AF, CHF</th>
<th>AF, CHF</th>
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<td>NYHA class (1–4)</td>
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<td>NAP‖</td>
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</table>

ACE indicates angiotensin-converting enzyme; AF, atrial fibrillation; CHF, congestive heart failure; EHRA, European Heart Rhythm Association; LV, left ventricular; NA, not available; NAP, not applicable; and NYHA, New York Heart Association.

*Receiving antihypertensive treatment.
†Hypoglycemic medication.
‡Lipid-lowering drug.
§Loop or thiazide diuretic.
‖Not applicable because CHF absent.
¶p<0.05, #p<0.001 vs controls without CHF or AF. Each AF group was compared statistically with the corresponding group without AF.
Table 2. Patient Characteristics: Tissue Samples

<table>
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<tr>
<th>Demographics</th>
<th>Sinus Rhythm (n=19)</th>
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<th>Chronic AF (n=8)</th>
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<td>Men, %</td>
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<td>44.4</td>
<td>37.5</td>
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<tr>
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<td>89.5</td>
<td>77.8</td>
<td>87.5</td>
</tr>
<tr>
<td>Hypertension</td>
<td>94.7</td>
<td>100.0</td>
<td>100.0</td>
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<td>Diabetes mellitus</td>
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<tr>
<td>Hyperlipidemia</td>
<td>73.7</td>
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<tr>
<td>Functional status</td>
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<tr>
<td>LV ejection fraction, %</td>
<td>51.6±3.6</td>
<td>44.3±6.1</td>
<td>46.6±7.6</td>
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<td>Concomitant medication, %</td>
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<td>21.1</td>
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<td>β-Blockers</td>
<td>73.7</td>
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<td>Lipid-lowering drugs</td>
<td>5.3</td>
<td>11.1</td>
<td>12.5</td>
</tr>
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</table>

ACE indicates angiotensin-converting enzyme; AF, atrial fibrillation; CAD, coronary artery disease; LV, left ventricular; and MVD/AVD, mitral valve disease/aortic valve disease.

*p<0.05 vs sinus rhythm, Fisher exact test.

described previously. Two unipolar leads were inserted into the right ventricular apex and connected to a pacemaker implanted in the neck. Twenty-four hours postoperatively, ventricular pacing was initiated at 240 bpm (sham dogs were followed for 1 week with deactivated pacemakers). After pacing, dogs were anesthetized with morphine (2 mg/kg IV, followed by 29.25 mg/kg per hour) and ventilated mechanically. The mean duration of 10-Hz burst pacing–induced AF was measured as an index of AF sustainability. Left atrial (LA) tissue was removed and rapidly frozen in liquid N₂. Tissue blocks were stored at −80°C for biochemical assays and/or were buffered in formalin for histological analysis. Fibrosis was evaluated with Masson trichrome staining and quantified by a blinded observer (percent cross-sectional area excluding blood vessels and perivascular tissue), as described previously. Atrial cells were isolated as described previously, and fibroblasts were separated from cardiomyocytes. Additional atrial samples were obtained from 8, 6, 6, and 5 dogs prepared for other projects in control, 24-hour, 1-week, and 2-week VTP groups, respectively, to obtain finer resolution of the time course of biochemical changes with VTP.

Mouse Model

Animal handling procedures followed National Institutes of Health guidelines (http://oacu.od.nih.gov/training/index.htm) and were approved by the Animals Research Ethics Committee of the Montreal Heart Institute. Eighteen mice (C57BL/6) weighing 15 to 20 g (from Charles River, Wilmington, MA) were allocated into either the green fluorescent protein (GFP) control adenovassociated virus group or the mir29b sponge virus group. Mice were anesthetized with isoflurane (2%). An adenovassociated virus, serotype 9, developed for cardiotoxic homing following peripheral injection, was used to transfer the mir29b sponge or a GFP control to mouse hearts via jugular vein injection to assess the in vivo consequences of mir29b downregulation. Two-hundred microliters of adenovassociated virus (2×10¹¹ to 9×10¹¹ viral genomes per 200 µL) was injected with a sterile 28-gauge needle. Mice were placed in individual cages and followed for 2 weeks. One GFP control mouse died during the follow-up period, leaving n= 8 for this group. After euthanasia (by cervical dislocation), the atria and left ventricle were removed and immediately frozen in liquid N₂ for quantitative polymerase chain reaction (qPCR) analysis (atria) or formalin-fixed for Masson trichrome staining (left ventricle).

RNA Extraction and qPCR

Canine RNA was extracted with Trizol, and human and mouse RNA was extracted with mirVana. RNA concentration was determined by nanodrop. cDNA was synthesized with 0.5 µg (mRNA quantification) or 10 ng of total RNA (microRNA quantification). qPCR was performed with TaqMan probes and primers or custom-made SYBR-Green probes (Table I in the online-only Data Supplement).

Collagen Protein Quantification

Fibroblasts were infected with lentiviruses (mir29b sponge or mir29b overexpressing) and grown for 5 days without changing of the medium. Medium was then removed for collagen assay by Western blot. Results were normalized to fibroblast number. Proteins were separated by electrophoresis on a 6% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked overnight with 3% bovine serum albumin and incubated with anti–collagen-I primary antibody, followed by secondary antibody conjugated to horseradish peroxidase. Signals were detected with chemiluminescence.

Manipulation of Fibroblast mir29b Expression

In vitro manipulation of mir29b expression, near-confluent (5–7 days) fibroblasts were trypsinized, counted, and plated in 12-well dishes at 100000 cells per well. After 4 to 6 hours for recovery, cells were infected with lentiviral constructs at 50 multiplicity of infection. Cells were kept in culture for 3 to 5 days after infection, and then samples were collected for qPCR by adding 500 µL per well of Trizol, followed by snap-freezing and storage at −80°C. We manipulated miR29b expression in fibroblasts with lentiviral-transfected gene constructs. To assess changes in canine atrial fibroblast target gene expression resulting from miR29b knockdown in vitro, we used viral gene transfer (Figure 4A) to deliver a sponge construct (miR29b sponge) along with GFP in a bicistronic vector. The sponge was designed to physically bind miR29b by complimentary base pairing, thereby causing its cellular degradation. Lentivirus overexpressing GFP only served as a negative control. We produced miR29b overexpression with a lentivirus overexpressing the primary miR29b sequence. A virus with a scrambled shRNA embedded in a primary miR29b context was used as a negative control. For in vivo manipulation, adenovassociated virus was used to deliver the knockdown sponge or GFP control (for details of adenovassociated virus construction, see Methods in the online-only Data Supplement).

Statistical Analysis

Data are presented as mean±SEM. For the time course analysis, plasma levels of patients, and atrial tissue of patients, 1-way ANOVA was used with a post hoc Dunnett test. An unpaired t-test was used for 2-group comparisons. Multivariable adjusted linear regression models were fitted to assess the associations between miR plasma concentrations in patients with AF and/or CHF while accounting for other predictors. Models were used that adjusted for (1) age and sex only; (2) coronary artery disease, hypertension, diabetes mellitus, and hypercholesterolemia in addition to age and sex; and (3) angiotensin-converting enzyme inhibitor or angiotensin II receptor type 1 blocker
Results

MiR29b Changes in a Canine CHF-Related AF Model

We first investigated potential miR29b changes in an animal model of AF-promoting, CHF-related structural remodeling. Hemodynamic indices measured during sinus rhythm are shown in Figure 1A through 1C. As reported previously,17,20 left ventricular end-diastolic pressure and left ventricular systolic pressure changed gradually (Figure 1A and 1B), whereas LA pressure increased at 1 week (Figure 1C). Masson trichrome stains (Figure I in the online-only Data Supplement) revealed progressive LA fibrosis, which became statistically significant 1 week after VTP onset (Figure 1D), concomitant with the appearance of an AF-maintaining substrate (Figure 1E).

Figure 2 shows results of miR29b LA expression studies. Total LA expression decreased by 87% within 24 hours and remained significantly decreased at 1-week and 2-week VTP (Figure 2A). Expression in cardiac fibroblasts largely paralleled that in whole atrial tissue, with a substantial decrease in miR29b at 24-hour VTP (−92% versus control; p<0.001) and with values remaining reduced subsequently (Figure 2B). Sham dogs had miR29 expression values that were not statistically different from those of controls (Figure II in the online-only Data Supplement), with mean values in fact greater than those of control dogs, excluding a role of surgery per se in the miR29 decrease seen in VTP dogs. Although we focused primarily on miR29b, because its copy number is double that of the miR29a and miR29c isoforms, we also measured the latter (which have the same target gene profile as miR29b) in canine atrial fibroblasts and found them to be significantly decreased (Figure III in the online-only Data Supplement), with a time course similar to that of miR29b (Figure 2B). We also measured the expression levels of miR590 and miR133a/b, which have been implicated previously in nicotine-induced atrial fibrotic remodeling.7 Whereas miR590 showed no significant changes in VTP dogs (Figure IVA in the online-only Data Supplement), the expression of miR133a (Figure IVB in the online-only Data Supplement) and miR133b (Figure IVC in the online-only Data Supplement) decreased significantly.

The majority of miR29b mRNA targets are ECM genes, including collagen 1A1 (Col1A1), collagen 3A1 (Col3A1), and fibrillin (FBN).3 Figure 3 shows the expression profiles of Col1A1, Col3A1, and FBN in isolated LA fibroblasts over time. Col1A1 gene expression (Figure 3A) roughly paralleled the development of fibrosis, increasing 9-fold at 1 week, with a further increase (15-fold) at 2 weeks. Col3A1 gene expression showed a similar pattern (Figure 3B). FBN expression also increased significantly at 2 weeks (Figure 3C).

Effects of Forced Alterations in miR29b Expression In Vitro

To directly assess changes in canine atrial fibroblast target gene expression resulting from altered miR29b expression in vitro, we used viral gene transfer to deliver a sponge construct designed to physically sequester miR29b by complementary binding to cause its cellular degradation (miR29b knockdown) and a miR29b-overexpressing virus to increase miR29b levels (schematic shown in Figure 4B). GFP was included in a bicistronic fashion to determine infection efficiency, which was nearly 100% (Figure 4A).

Sponge-bearing virus infection decreased fibroblast miR29b expression by 68% (Figure 4C). This miR29b decrease was accompanied by significant increases in Col1A1 (+28%), Col3A1 (+19%), and FBN (+20%) mRNA expression (Figure 4C, left). COL1A1 protein was measured from the supernatant of gene-transferred fibroblasts and was found to increase by ≈90% in miR29b knockdown cells versus GFP controls (Figure 4C, right). The miR29b-overexpressing virus increased miR29b expression by 3-fold compared with a scrambled control virus (Figure 4D). MiR29b overexpression significantly reduced Col1A1, Col3A1, and FBN mRNA expression by 65%, 62%, and 61%, respectively (Figure 4D, right).
left). COL1A1 protein in the supernatant of fibroblasts overexpressing miR29b was decreased by ≈60% (Figure 4D, right).

**MicroRNA Plasma Concentrations and Atrial Tissue Expression in Humans**

Because plasma levels of miRs are known to change in various diseases, we investigated miR29b changes in patients. Plasma miR29b levels were reduced significantly, by ≈50%, in both AF and CHF patients versus controls. When AF patients were subdivided into those with versus without CHF, miR29b expression was similar in patients with AF only and CHF only but was lower with both conditions concomitantly (Figure 5A). MiR21 plasma levels were also decreased in patients with AF (−61%) and CHF (−64%) versus non-CHF, non-AF controls (Figure 5B), but there was no significant difference for patients with AF and CHF versus controls. No significant differences among groups were seen for miR133a and miR15 among control, CHF, and CHF/AF groups when all potential contributors were considered in model 3.

We then examined miR concentrations in right atrial appendage tissues from patients with or without AF. MiR29b tissue expression was decreased by 40% in paroxysmal AF patients and by 54% in chronic AF patients versus sinus rhythm patients (Figure 6A). MiR133a expression was similar in all groups (Figure 6B).

**Manipulation of miR29b In Vivo With a Mouse Model**

To assess directly the effect of decreased miR29b expression in vivo on collagen expression, a miR29b sponge carried by a cardiotropic adenoassociated virus (AAV-9) was used. Mice were injected systemically with either a GFP control construct or a sponge construct to decrease miR29b expression. Good myocardial infection was obtained with both (Figure V in the online-only Data Supplement). Atrial COL1A1 mRNA levels increased by ≈45% in the sponge group compared with GFP control virus–injected mice (Figure 7A). Tissue collagen protein expression was assessed with Masson trichrome staining (Figure 7B and 7C). Collagen content was approximately doubled in miR29b sponge–exposed hearts (Figure 7B).

**Discussion**

In this study, we demonstrated a rapid decrease of miR29b atrial expression in a canine CHF model of atrial fibrosis associated with an AF-maintaining substrate, followed by increased expression of miR29b ECM target genes, in CHF (p=0.13) or the combination of CHF and AF (p=0.27). No statistically significant differences were seen for miR133 or miR15 among control, AF, CHF, and AF/CHF groups when all potential contributors were considered in model 3.
particular the collagen components (Col1A1 and Col3A1) that constitute >90% of cardiac fibrotic tissue. Manipulation of miR29b expression in the target cells (fibroblasts) produced changes consistent with an important collagen regulatory role. We further demonstrated that plasma levels of miR29b are decreased in AF and CHF patients, with further decreases in patients with both, even after accounting for potential confounding factors. In addition, atrial tissue levels of miR29b were decreased in AF patients. Mimicking CHF-induced decreases in miR29b expression with cardiac homing adenoassociated viral knockdown gene transfer in the mouse confirmed collagen upregulation.

**Comparison With Previous Studies of Profibrotic MicroRNAs**

Recent studies have pointed to a role for miR21, miR133a, and miR29b in tissue fibrosis. MiR21, upregulated via Smad3 signaling, promotes renal fibrosis. Smad3-deficient mice show increased miR29b levels and no fibrosis after obstructive nephropathy. MiR21 is upregulated in idiopathic pulmonary fibrosis, and antisense miR21 diminishes fibrosis in mice. The role of miR21 in cardiac fibrosis is controversial. Thum et al showed that inhibiting miR21 suppresses fibrosis in a mouse model of pressure overload. However, Patrick et al showed that miR21 knockout mice display unchanged responses (including fibrosis) to cardiac stress. In a recent study, we found that miR21 knockout with anti-miRs in post-MI rats decreases atrial fibrosis and AF duration.

MiR133 is muscle specific, with relatively weak expression in fibroblasts, and plays a major role in cardiomyocyte hypertrophy. Transforming growth factor-β contains multiple putative binding sites for miR133, and miR133 downregulation contributes to increased transforming growth factor-β levels. MiR133 also binds to and inhibits connective tissue growth factor, which has profibrotic properties. Whereas atrial fibroblast miR133 levels decreased in VTP dogs, atrial miR133 expression was unchanged in AF patients.
Downregulation of miR29 has been identified as potentially pathogenic in a mouse model of hepatic fibrosis, and miR29b is downregulated in patients with liver disease. Similarly, miR29b is inversely correlated with pulmonary fibrous tissue content in a mouse model of lung fibrosis. MiR29b is also dysregulated in mice with experimental MI. In right atrial appendage samples from mitral stenosis patients with AF, members of the miR29 family are downregulated (miR29a, −1.54; miR29b, −1.42; miR29c, −1.27 versus control), as is miR133a (−1.58 versus control). MiR29b was also shown to increase with age in the aorta of mice and humans and was increased in patients with aortic aneurysm compared with control.

On the basis of the biology of miR29b and the fact that it was consistently decreased in the atria and plasma of our AF patients, miR29b plasma levels were measured in control (without [w/o] congestive heart failure [CHF] and without atrial fibrillation [AF]; w/o CHF w/o AF); patients with (w) AF only (w AF w/o CHF); patients with CHF only (w CHF w/o AF); and patients with both CHF and AF (w CHF w AF). A, miR29b plasma levels. B, miR21 plasma levels. C, miR133a plasma levels. D, miR15 plasma levels. ***p<0.001, **p<0.01, *p<0.05 vs CTL, 1-way ANOVA followed by Dunnett post hoc test. Numbers indicate patients per group. Data are mean±SEM.
patients, we decided to fully investigate the role of miR29b in animal models. Changes in atrial miR29b expression occurred early in the pathophysiological process induced by VTP in dogs, when systolic pressure had decreased but cardiac filling pressures had not yet changed, and preceded collagen gene upregulation. These findings, along with the demonstration that miR29b expression directly regulates collagen genes in canine atrial fibroblasts and that in vivo miR29b knockdown enhances cardiac collagen expression in the mouse, suggest a role for miR29b in CHF-related atrial fibrosis and the associated enhancement in AF sustainability. Consistent with our findings in canine CHF, miR29 was also downregulated in CHF patients.

**MicroRNAs as Biomarkers**

Recent studies have highlighted the potential value of microRNA biomarkers for heart disease. Circulating levels of miR126, miR17, miR92a, and miR155 are decreased in patients with coronary artery disease. In addition, miR1 and miR499 show increased expression in post-MI patients. Patients with heart failure have increased blood levels of several microRNAs. Of these, only miR423-5p was significantly and independently correlated with the diagnosis of CHF. In their screening microarray, miR29b concentrations were reduced by $\approx 33\%$ relative to control, with borderline unadjusted statistical significance ($p=0.052$). Plasma levels of miR29b, miR21, and miR133a were unchanged in patients with diastolic dysfunction. MiR150 was also shown to be decreased in AF patients versus healthy controls and was independently associated with AF. Interestingly, changes in circulating microRNAs precede MI occurrence, with altered expression of 3 microRNAs associated with MI: miR216, miR223, and miR197. We chose to focus on miRs involved in fibrosis. MiR133a was unchanged in both tissue and plasma of AF patients. MiR21 concentrations were decreased in plasma of AF and CHF patients, but after correction for associated conditions and drug therapy, they were no longer significantly different in AF patients. MiR29 showed the clearest and most coherent changes in both plasma and atria of AF patients and was also found to play a potentially significant role in collagen regulation. Extensive clinical studies will be needed to establish the potential value of miR29b and other candidate miRs in predicting the natural history and guiding management of AF. Further studies are also needed to understand the determinants of circulating miR concentrations and the manner in which they can aid in disease management and prevention.

**Potential Mechanisms of miR29b Downregulation**

Transforming growth factor-β has been implicated in miR29b downregulation and is upregulated in canine atrium after 24-hour VTP. This timing corresponds to the onset of miR29b downregulation in the present study. However, transforming growth factor-β expression returns toward normal during continued VTP, whereas in the present study miR29b expression remained decreased for at least 2 weeks, implicating additional mechanisms. A recent study pointed to a role of nuclear factor-κB (NF-κB) in miR29b downregulation. There are 4 NF-κB binding sites near the transcriptional start site for miR29b; NF-κB activation represses promoter activity. NF-κB expression is increased in fibrotic myocardium of CHF patients, and NF-κB is activated in the atria of AF patients. NF-κB involvement may also contribute to the atrial-selective fibrosis (versus ventricular) commonly seen with cardiac pathology.

**Potential Significance**

Structural remodeling is an important paradigm in AF. To our knowledge, our study is the first to report a potential role for miR29b in AF-related atrial profibrotic remodeling. Van Rooij et al provided the first evidence indicating involvement of miR29b in the ventricular profibrotic response after MI. They found miR29 to be selectively expressed in cardiac fibroblasts and to be downregulated in the MI border zone. They demonstrated regulation of ECM gene expression by miR29b, implicating miR29b in the formation of post-MI scar and border zone fibrosis. The ability of miR29b overexpression to suppress collagen expression in atrial fibroblasts (Figure 4) points to the potential value of miR29 manipulation in influencing the development of the AF substrate.

Our study also points to miR29 as a potential AF plasma biomarker. Although much remains to be learned about circulating microRNAs, it has been shown that they are carried by microparticles and exchange information between cells. There is great interest in noninvasive markers of atrial structural remodeling that could provide prognostic information and be helpful in assessing preventive approaches. MiR29b, because of its potential pathophysiological significance, merits consideration, along with other recently described biomarkers in AF such as brain natriuretic peptide.

**Potential Limitations**

We chose to focus on miR29b because of its important role in post-MI ventricular fibrosis and the consistent decrease of miR29 expression in the atria and plasma of AF patients. It is unlikely that a single microRNA is the sole determinant of structural remodeling in complex conditions such as AF or CHF. Indeed, the expression of a substantial number of microRNAs is altered in CHF and may be pathophysiologically important. MiR21 appears to play an important role in post-MI atrial fibrotic remodeling in rats, and other candidates include miR133 and miR590. The results of the present study indicate for the first time that miR29b is a strong candidate to play a pathophysiological role in atrial structural remodeling and is also an interesting candidate AF biomarker. MiR29b was significantly downregulated at 24 hours, whereas significant fibrosis was apparent at 1 week. These results indicate a delay from miR29b downregulation to collagen accumulation. Some of this lag likely reflects the delay from downregulation of miR29b expression to the consequent upregulation of collagen gene expression, as well as the time needed for detectable collagen protein accumulation. Fibrous tissue content was nonsignificantly increased by $\approx 65\%$ at 24 hours, and a highly significant, 5-fold increase was apparent at 1 week. Therefore, collagen content increased some time between 1 day and 1 week, with a lag not incompatible with a causative role for miR29b. In addition, other microRNAs and signaling systems may have been involved.

We used a specific animal model of CHF-associated AF. Extrapolation to clinical forms should be cautious, although
it is intriguing that changes in miR29b plasma and tissue concentration of AF and CHF patients mirror our observations in the experimental model. We chose a lentiviral expression vector for in vitro miR29b expression manipulation because of its long-lasting expression and improved nuclear targeting. However, there are other potentially effective means of modifying microRNA expression, including delivery of highly stable, chemically modified microRNA analogues. In addition, we were able to achieve effective regulation of cardiac collagen expression with peripheral vein injection of an adenovassociated viral vector bearing a microRNA knockdown construct, and therefore cardiac-specific modulation of miR29 may be achievable with systemic administration. We tried to quantify miR29b knockdown by the sponge virus; however, this proved to be impossible because our qPCR probes cross-reacted with a component of the virus. We were unable to design alternate probes because of the very short length of miR29b.

MiR29 exists as 3 isoforms (a through c). Consistent with the bicistronic coupling of miR29b with miR29a and miR29c, the 3 miRs form a single miR29 family with consistent expression and biology. Accordingly, we saw changes in cardiac fibroblast expression of miR29a and miR29c in VTP dogs (Figure III in the online-only Data Supplement) similar to those that occurred with miR29b. We focused on miR29b because its gene copy number is twice that of miR29a and miR29c analogues (which have identical targeting) and because it is the most strongly expressed isoform in fibroblasts.

Conclusions

In an animal model of AF associated with VTP-induced CHF, miR29b levels are rapidly and strongly downregulated. Atrial fibroblast expression of the ECM genes COL1A1, COL3A1, and FBN is regulated by miR29b, and in vivo miR29b knockdown increases cardiac collagen expression. Plasma and atrial tissue samples from AF patients show decreased miR29b expression. Therefore, miR29b appears to be important both in terms of generation of the AF substrate and as a candidate biomarker for atrial remodeling. Restoring miR29 expression may be a potentially interesting approach to suppressing AF-promoting structural remodeling.

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Disclosures

None.

References

miR29 shows promise as an AF biomarker and is a potential therapeutic target for AF prevention. We found that atrial miR29 is rapidly downregulated in dogs: atrial remodeling of a different sort.

**REFERENCES**


MicroRNA29: A Mechanistic Contributor and Potential Biomarker in Atrial Fibrillation
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SUPPLEMENTAL MATERIALS

MicroRNA29: A Mechanistic Contributor and Potential Biomarker in Atrial Fibrillation

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Supplemental Methods

Canine animal model

All animal handling procedures followed National Health Institute guidelines (http://oacu.od.nih.gov/training/index.htm) and were approved by the Animals Research Ethics Committee of the Montreal Heart Institute. Ninety-four mongrel dogs (22-36 kg) were divided into 5 groups: control (n=32), sham (n=5), 24-hour VTP (n=19), 1-week VTP (n=16), and 2-week VTP (n=22). VTP-dogs were chronically-instrumented as previously described under diazepam (0.25 mg/kg IV)/ketamine (5.0 mg/kg IV)/halothane (1% to 2% PI) anaesthesia. Sham-dogs were instrumented like VTP dogs and followed for 1 week with pacemakers deactivated. Two unipolar leads were inserted into the right-ventricular apex and connected to a pacemaker implanted in the neck. Twenty-four hours post-operatively, pacemakers were
programmed to pace the ventricles at 240 bpm. After the pacing intervals, dogs were
anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV, followed by
29.25 mg/kg/h) and ventilated mechanically. Hemodynamic measurements were obtained with
fluid-filled catheters and pressure-transducers. The duration of 10 Hz-burst-induced AF
(irregular atrial rhythm >400/min) was measured repeatedly as previously described\(^1\) to measure
mean AF-duration as an index of AF-sustainability. Hearts and adjacent lung tissues were then
excised via median thoracotomy and immersed in oxygenated Tyrode solution for further tissue
preparation. Left atrial (LA) tissue was removed and rapidly-frozen in liquid-N\(_2\). Tissue blocks
were stored at -80°C for biochemical assays and/or were buffered in formalin for histological
analysis. Additional atrial samples were obtained from 8, 6, 6 and 5 dogs prepared for other
projects in control, 24-hour, 1-week and 2-week VTP groups respectively, in order to obtain finer
resolution of the time-course of selected biochemical changes, particularly miR29b expression,
with VTP.

**Fibrosis assessment**

Fibrosis was evaluated with Masson’s trichrome staining. LA-sections from the free-wall and
from the LA-appendage were cut into 5-µm sections along longitudinal and transverse planes.
Fibrosis was quantified by a blinded observer as previously described\(^2\) and expressed as %
cross-sectional area, excluding blood vessels and perivascular tissue. Results were averaged for
each dog for statistical analysis.

**Fibroblast isolation**

Atrial cells were isolated as described previously.\(^3\) Cells were then centrifuged at 800 rpm for
2 minutes to pellet cardiomyocytes. The supernatant was filtered through 0.2-µm mesh to
remove any remaining cardiomyocytes and then centrifuged at 2,000 rpm for 10 minutes to pellet fibroblasts. The remaining supernatant was removed, 500-µL Trizol added and samples snap-frozen in liquid-N₂ and stored at -80°C for RNA-extraction and qPCR. These samples are referred to as freshly isolated fibroblasts.

**RNA extraction and qPCR from canine atrial tissue**

LA tissue samples were kept in liquid nitrogen and 30-50 mg of tissue from each sample was removed and pulverized using a mortar and pestle. One mL of Trizol (Invitrogen) was immediately added to each sample and the samples were homogenized using a Polytron device. 0.2 mL of chloroform was added to each sample and the manufacturer’s protocol was followed to isolate RNA. After drying the pellet, total RNA was dissolved into 30 µL of RNase free water (Hyclone). The concentration of RNA was determined by nanodrop (NanoDrop, Wilmington, DE). Total RNA (0.5 µg for non-miRNA and 10 ng for miRNA) was used to synthesize cDNA with a High Capacity cDNA Reserve Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. cDNA samples (2 µL for mRNA and 1.33 uL for miRNA) were used for real time PCR in a total volume of 20 µL using Taqman Universal Master Mix (Applied Biosystems) and qPCR was performed with TaqMan probes and primers or custom-made SYBR Green probes (Applied Biosystems) see Supplemental Table 1 for detailed primer information. Fluorescence signals were detected in duplicate by qPCR (Stratagene Corp, La Jolla, Calif). miR29b from tissue was normalized to HPRT, and miR29b from freshly isolated fibroblasts was normalized to the geometric mean of 4 reference genes: U6, HPRT, beta-actin and 18S. mRNA was referenced to beta-2-microglobulin in freshly isolated fibroblasts. All genes measured in the *in vitro* experiments were normalized to the geometric mean of 4 reference genes: HPRT, beta-
actin, U6 and 18S. Each gene was detected in duplicate and analyzed with the delta-CT method. Data was quantified using MxPro qPCR software.

**Plasmid constructs**

**Mir29b knock-down**

The lentiviral vector plasmid which encodes the mir29b decoy tag linked to GFP mRNA was generated as follows. Partially overlapping complementary synthetic oligonucleotides (sense: 5’ TAGCGGCCGCTGCTGTGGACAACAACACTGATTTCCTGTGGTGCTAAACACTGATTTCCTGTGGTGCT, antisense: 5’ ATACGCGTTCCGAGGCAGTTAGCTTATGTCCCTGATGTTGATAGCTTATGTCCCTGATGTGTTGA) were PCR-amplified. Both the sense and antisense template strands carry bulged target sites for mir29b (one target site is underlined as an example) and NotI and MluI restriction sites (italicized) at their 5’ end, respectively. The PCR was carried out in a 50 µL final volume reaction, the reaction mixture contained 1x Taq polymerase buffer (Invitrogen), 5% DMSO, 5 mM MgCl₂, 200 µM dNTP, 500 nM sense primer (5’ TAGCGGCCGCTGCTGTGGAC), 500 nM antisense (5’ ATACGCGTTCCGAGGCAGT), 25 ng of each template strand and 3 U Taq DNA polymerase (Invitrogen). The temperature protocol was (94°C 1 min, 65°C 30 s, 75°C 1 min) x 1, (94°C 30 s, 65°C 30 s, 75°C 30 s) x 24, (94°C 30 s, 65°C 30 s, 75°C 11 min) x 1. The PCR product was cloned into the pGIPZ lentivirus vector plasmid (Open Biosystems) at the unique NotI and MluI sites following standard laboratory practices. Sequence identity of the resulting plasmid was verified by sequencing.

As a negative control for mir29b knock-down we used a virus consisting of the pGIPZ vector plasmid without modification (Open Biosystems).
**Lentiviral-mediated mir29b overexpression**

The pLemir pri-mir29b over-expressing lentiviral vector plasmid was obtained from Open Biosystems. The sequence-verification of the clone revealed that the purchased plasmid carried the pri-mir29b sequence in the opposite orientation with respect to the direction of transcription driven by the CMV promoter of the plasmid. The sequence analysis also showed that the pri-mir29b sequence can be mobilized by NotI and XhoI restriction enzymes with minimal flanking sequences. We sub-cloned the NotI-XhoI fragment of pLemir carrying the pri-mir29b sequence in pGIPZ at its unique NotI-XhoI sites, which resulted in a CMV-GFP-pri-mir29b construct with the correct orientation of pri-mir29b.

As a negative control for the mir29b over-expressing virus, a virus over-expressing a scrambled shRNA in a pri-miRNA context (shRNAmir) was used. The scrambled shRNAmir over-expressing plasmid was generated as follows. The empty pGIPZ lentivirus vector plasmid carrying the mRNA-context sequence but no shRNA was obtained from Open Biosystems. The EcoRI site of pGIPZ located at position 5394 was removed by partial EcoRI digestion and Klenow fill-in, resulting in pGIPZΔEcoRI. This modification allowed the direct cloning of the scrambled shRNAmir construct in pGIPZ between XhoI, 2654 and EcoRI, 2678 sites. In the design of the scrambled shRNAmir and during the rest of the cloning procedure we followed protocols in.\(^4\) Briefly, a 97bp long synthetic oligonucleotide (5` TGCTGTGGACAGTGAGCGCAGTATCAGACAGATAATGAATAGTGAGCCACAGATGT 3`, **passanger strand**, loop, **guide strand**) was PCR amplified by 5`

CAGAAGGCCTGAGAAGGTATATTGCTGTTGACAGTGGAGCG sense and 5`

CTAAAGTAGCCCCTTGAATTCCGAGGAGTGGCA antisense primers, carrying XhoI and
EcoRI restriction sites, respectively. PCR product was cloned in pGIPZΔEcoRI at XhoI, EcoRI sites. Sequence identity of the resulting clone was verified by sequencing.

**Lentivirus packaging plasmids**

The psPAX2 and pMD2.G lentivirus packaging plasmids were obtained from Didier Trono’s laboratory (http://tronolab.epfl.ch, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) through Addgene (Addgene plasmid 12260 and 12259).

All plasmids were amplified in *E. coli* DH5α and purified by Nucleobond anion exchange columns (Macherey-Nagel, Bethlehem, PA, USA) following the manufacturer’s instructions.

**Lentivirus production**

The Hek293T/17 cell line we used for producing lentivirus production was obtained from ATCC (Manassas, VA, USA) and were grown in DMEM (Invitrogen) supplemented with 10% FCS (Gibco). Lentiviruses were produced by following the protocols available from Didier Trono’s laboratory (http://tronolab.epfl.ch) with minor modifications. A subconfluent monolayer of Hek293T/17 cells (ATCC) kept in DMEM (Invitrogen) supplemented with 10% FBS (Gibco) was transfected with a mixture of plasmids containing one of the vector plasmids and the psPAX2 and pMD2.G packaging plasmids in 2:2:1 weight ratio by the calcium-phosphate precipitation method. Eight hours after transfection, the culture medium was replaced by fresh culture medium. The supernatant containing the lentivector particles was harvested two times 32 and 56 hours post-transfection. The harvested medium was clarified from cell debris by low-speed centrifugation and by filtration through a 0.45 µm pore size syringe-attached filter. Virus particles were concentrated by ultracentrifugation at 47000 g (RCF average) for 2 hours in
a swinging bucket rotor, re-dissolved in sterile PBS containing 1% BSA and stored at -80°C in aliquots. Virus preparations were titrated on Hek293T/17 cells.

**In vitro manipulation of fibroblast miR29b expression**

LA fibroblasts were isolated as described previously$^5$ with the exception that DMEM (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin was used instead of Medium 199. At near-confluence (5-7 days) fibroblasts were trypsinized, counted, and plated in 12-well dishes at 100,000 cells/well. After 4-6 hours for recovery, cells were transfected with lenti-viral constructs at 50 m.o.i. Cells were kept in culture for 3-5 days post-infection and then samples were collected for qPCR by adding 500 µL/well Trizol followed by snap-freezing and storage at -80°C.

MiR29b knock-down was achieved with a lentivirus over-expressing a competitive inhibitor sequence of mir29b (mir29b-sponge) coupled to GFP mRNA. Lentivirus over-expressing GFP only served as a negative control. Forced miR29b-over-expression was obtained with a lentivirus over-expressing the pri-mir29b sequence. As a negative control in the over-expression experiments we used a virus with a scrambled shRNA embedded in a pri-miRNA context.

**Adenoassociated-virus constructs**

For *in vivo* mir29b-sponge delivery, we used a self-complementary adenoassociated virus-based vector system. The scAAV-GFP vector plasmid was a kind gift from K. Reed Clark (Addgene plasmid number 21893).$^6$ The sponge sequence was PCR amplified from a synthetic oligonucleotide template (5’ CAAGTAAGTCTAGATGCTGTTAACACTGATTTCTGTGGTGCTAACAATGATTCTGTGCTGCTAACAACACTGATTTCTGTGGTGTGCTAGAATCTAGTGC
GC) encoding 3 mir29b bulged target sites, with 5’ GCTGTACAAAGTAAGTCTAGATGCTGTT sense and 5’ TAGCGGCCGCACTAGTTTC antisense primers. The consequent PCR-product carried BsrGI and XbaI recognition sites on the 5’ end and SpeI and NotI sites on the 3’ end. The PCR product was double-digested with BsrGI/SpeI and XbaI/NotI in separate reactions and a three-way ligation was carried out, which contained BsrGI/NotI digested scAAV-GFP plasmid and BsrGI/SpeI and XbaI/NotI digested PCR-product in equal molar ratios. The resulting scAAVsp29b plasmid carries a GFP reporter-gene driven by the EF1 promoter and 6 mir29b target sites in the 3’ UTR of GFP; its integrity was verified by sequencing.

The pDG9 packaging plasmid providing adenovirus helper functions and adenoassociated virus serotype-9 capsid was a kind gift from Roger Hajjar (Cardiovascular Research Center at Mount Sinai School of Medicine).

**Adenoassociated-virus production**

Hek293T/17 cells were grown in 10-cm diameter tissue-culture dishes in DMEM, supplemented with 10% serum. Subconfluent dishes were transfected with 4 µg of vector plasmid and 12 µg of pDG9 packaging-plasmid in the presence of 64 µg polyethyleneimine (25 kDa, linear, Polysciences Inc.). Cells were harvested 48-72 hours post-transfection and were lysed by repeated freeze-thaw cycles. Lysates were treated with Bensonase nuclease and were separated on iodixanol density-gradients as described by Zolotukhin et al. The AAV fractions collected from the 25% iodixanol layer were desalted and formulated by using the Amicon Ultra centrifugal filter units (100 kDa, Millipore) into Ringer’s lactate solution. Virus vector preparations were aliquoted and stored at -80°C.

Genome copy numbers of AAV vector preparations were determined by real-time qPCR using the 5’ ACGACGGCACTACAAGA sense and 5’ TGCTTGTCGGCCATGATA antisense
primers specific for the eGFP ORF carried by both the mir29b-sponge and the GFP control virus. Standard curves were constructed from serial dilutions of the scAAV-GFP plasmid and were run in parallel with the unknown AAV vector preparations. AAV vector genome copy numbers were determined by interpolating from the standard curve.

**In vivo manipulation of mouse cardiac miR29b expression**

Animal-handling procedures followed National Institutes of Health guidelines (http://oacu.od.nih.gov/training/index.htm) and were approved by the Animals Research Ethics Committee of the Montreal Heart Institute. Eighteen mice (C57BL/6) weighing between 15-20 g (from Charles River, Wilmington, MA, USA) were allocated into either the GFP-control adenoassociated-virus group or the miR29b-sponge virus group (9/group). One GFP-control mouse died during the follow-up period, leaving an N of 8 for this group. Mice were anesthetized with isoflurane (2%) and hair around the neck was chemically removed to allow visual identification of the jugular vein. Two-hundred µL of adenoassociated virus, serotype 9 (2x10^{11} – 9 x10^{11} vg/ 200 µL, Supplemental Table 3) were injected systemically with a sterile 28 gauge needle and an insulin syringe (Becton, Dickinson and Company; Franklin Lakes, NJ) via the jugular vein. Mice were placed in individual cages and followed for 2 weeks. Following euthanasia (by cervical dislocation) the heart was dissected into the atria, left ventricle, and right ventricle. Tissue was immediately frozen in liquid-N_{2}. RNA was extracted with miRVana (Ambion) and used for qPCR analysis (atria) and Masson Trichrome staining (left ventricle). Collagen quantification by Masson Trichrome staining could not be performed on atrial tissue because the very small mass of the mouse atrium and the need to use atrial samples for qPCR analysis.
**Quantification of collagen content by Western blot**

Fibroblasts were infected with lentiviruses (miR29b-sponge or miR29b over-expressing) as described above. Twenty-four hours post-infection, a medium-change was performed and 1 mL of medium was added per well of a 12-well dish. Fibroblasts were then allowed to grow for 5 days without changing medium. After this 5-day period, the medium was removed and immediately frozen in liquid-N₂. Fibroblasts were trypsinized and counted. Since collagen is a secreted protein and most collagen that is produced moves into the extracellular space, collagen was assayed in the medium and results normalized to cell number per well.

For collagen-detection, a 6% SDS-PAGE gel was loaded with supernatant mixed with loading buffer at a maximum volume of 60 µL and a minimum of 54 µL. Proteins were separated by electrophoresis followed by transfer to nitrocellulose membranes. Membranes were blocked overnight with 3% bovine serum albumin and incubated for 1 hour at room temperature with primary antibody to collagen-I (1/20,000 MD Sciences, MD20151). After membranes were washed, they were incubated with anti-rabbit secondary antibody (1/10,000, Jackson) conjugated to horseradish peroxidase for 1 hour at room temperature. Signals were detected with chemiluminescence and quantified with Biorad Quantity One software.

**Determination of miRNAs in plasma of AF patients**

**Patients**

To further investigate a potential role of miRNAs as a biomarker in AF patients we investigated miRNA plasma levels in AF patients (n=33). Thirty-three patients followed for persistent AF constituted the AF-population (patients with permanent or paroxysmal AF were excluded). AF classification was performed according to the AHA/ACC guidelines for the management of patients with atrial fibrillation and AF was considered persistent when the arrhythmia sustained
beyond 7 days and termination with pharmacological therapy or direct-current (DC) cardioversion did not fail >2 consecutive times. In addition, a EHRA AF symptom score was assessed at admission according to ESC guidelines for management of atrial fibrillation (EHRA score: EHRA I “no symptoms”; EHRA II “mild symptoms”, normal daily activity not affected; EHRA III “severe symptoms”, normal daily activity affected; EHRA IV “disabling symptoms”, normal daily activity discontinued). Patients were included when the initial ECG recording at admission showed AF (an irregular atrial rhythm with no discernable P waves in the ECG), which persisted ≥7d; patients had to be in a compensated clinical cardiac and pulmonary state and show heart rate of 50-100 bpm (during AF) at the time of blood sampling. Blood samples were taken while the patients were still in AF. In addition, thirty-two patients with CHF and no history of AF as ascertained by ECG on at least 3 clinic visits constituted the CHF-only population. The diagnosis of CHF was based on clinical criteria according to the ACC/AHA 2005 guidelines for the diagnosis and management of heart failure in adults, with typical clinical symptoms (NYHA class II-III) and a reduced left-ventricular ejection fraction on echocardiography. Patients with decompensated heart failure (NYHA class IV) were excluded. The AF group was subdivided into patients with (n=16) and those without (n=17) CHF based on the same clinical criteria. Controls were patients from the surgical outpatient clinic (n=30), in whom heart disease had been excluded by ECG and clinical examination. In addition, we excluded control patients with chronic inflammatory diseases, history of cancer, liver disease or neurodegenerative disease. Following Mitchell et al. we measured miRNA in plasma.

**Blood processing**

Blood samples from patients were obtained with informed consent under institutional review board-approved protocols. Blood of all subjects was collected via a direct venous puncture into
9 ml EDTA containing tubes (Sarstedt Monovette). All blood was processed for isolation of plasma within 4 hours of collection. Blood was processed by spinning at 4000 rpm for 20 minutes at room temperature. Plasma was carefully transferred to a fresh RNase/DNase free tube and stored at -80°C.

**RNA Isolation**

Total RNA was isolated using the miRNeasy kit (Qiagen) and a modified protocol based on Dimmeler et al.\(^\text{12}\) Plasma was thawed on ice and 400 µL EDTA-plasma was mixed with 4000 µL TRIzol (Invitrogen), incubated for 5 minutes at room temperature and subsequently mixed with 800 µL chloroform. The organic and aqueous phase was separated by centrifugation. The aqueous phase containing the RNA was carefully removed and RNA was precipitated by addition of 100% ethanol. The mixture was applied to a RNeasy Mini spin column, washed several times and RNA was eluted by addition of 35 µL RNase-free water (95°C).

To date, no housekeeping miRNA has been established and validated to normalize for the miRNA content. Therefore, we chose to use a fixed volume of plasma per sample and a synthetic *Caenorhabditis elegans* miR-39 (cel-miR-39, 20 fmol/sample, synthesized by Qiagen) as a spiked-in control to normalize for individual RNA-isolation-related variations. Twenty fmol cel-miR-39 were introduced to each plasma sample after addition of denaturating Qiazol solution. For each RNA sample, the *C. elegans* spiked-in miRNAs were measured using TaqMan qRT-PCR assays (Applied Biosystems).

**Measurement of miRNA Levels in Plasma with TaqMan qPCR Assays**

A fixed volume of diluted RNA (5 µL) was subjected to reverse transcription using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s
Subsequently, 1.33 µL of the product was used to detect miRNA-expression by quantitative PCR using miRNA-specific stem-loop primers (Applied Biosystems) for the corresponding microRNA. Quantitative PCR reactions were performed on a Bio-Rad iQ5 system using the following program: 10 minutes pre-incubation at 95°C, 40 cycles of 15 seconds denaturation at 95°C and 60 seconds of elongation at 60°C. Values are normalized to cel-miR-39 and expressed as $2^{-[(CT \text{ microRNA)}-(CT \text{ cel-miR-39)]]}$.

**Statistical analysis**

Data are presented as mean±SEM. For the time-course analysis, atrial tissue of patients and plasma levels of patients, one-way ANOVA was used with a post-hoc Dunnett’s test. An unpaired t-test was used for two-group comparisons. Multivariable adjusted linear regression models were fitted to assess the associations between miR-plasma concentrations in patients with AF and/or CHF compared with controls, while accounting for other predictors. Models were used that adjusted for (1) age and sex only; (2) coronary artery disease, hypertension, diabetes mellitus, and hypercholesterolemia in addition to age and sex; and (3) angiotensin converting enzyme inhibitor or AT1-receptor blocker treatment, use of aldosterone antagonists, and use of statins in addition to the factors included in model (2). Categorical variables are analyzed by Fisher’s exact test with Bonferroni correction. A two-tailed $P<0.05$ was considered statistically-significant.
References


### Supplemental Table 1A. qPCR primer information: Taqman Probes from Applied Biosystems

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<tr>
<td>Hsa-miR-29b</td>
<td>Target miR</td>
<td>000413</td>
<td>MI0000105</td>
<td>UAGCACCAUUUGAAAAUCGAGUGUU</td>
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<td>Hsa-miR29c</td>
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<td>Hsa-miR15b</td>
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<td>000390</td>
<td>MI0000438</td>
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<tr>
<td>Hsa-miR21</td>
<td>Target miR</td>
<td>000397</td>
<td>MI0000077</td>
<td>UAGCUUAUCAGACAGUGUAA</td>
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<td>Hsa-miR590</td>
<td>Target miR</td>
<td>001984</td>
<td>MI0003602</td>
<td>GAGCUUAUUCAUAAAGAGGACG</td>
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<td>Hsa-miR133a</td>
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<td>000458</td>
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<td>Hsa-miR133b</td>
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<td>002247</td>
<td>MI0000822</td>
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<tr>
<td>U6 snRNA</td>
<td>Reference miR</td>
<td>001973</td>
<td>NR_004394</td>
<td>GTGCTCGCTTCCGAGCAGCACATATATACAAATTTGGTAGTATTAGCATGCGCTGCTGCAAGGATGACAGCAGCACCCGATGTCGTTCCATATTTT</td>
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<tr>
<td>Collagen 3A1</td>
<td>Target gene</td>
<td>Cf02631369_m1</td>
<td>--</td>
<td>--</td>
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<tr>
<td>(COL3A1)</td>
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<tr>
<td>Fibrillin 1</td>
<td>Target gene</td>
<td>Cf02667257_m1</td>
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<td>(FBN1)</td>
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<tr>
<td>hypoxanthine phosphoribosyl transferase 1 (HPRT1)</td>
<td>Reference gene</td>
<td>Cf02626258_m1</td>
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<tr>
<td>Collagen 1A1</td>
<td>Target gene</td>
<td>Custom made</td>
<td>--</td>
<td>Fwd primer – 5’ CCAAGAGGAGGGCCAAGAA</td>
</tr>
<tr>
<td>(COL1A1)</td>
<td></td>
<td>Canis lupus familiaris</td>
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<td>Reverse primer – 5’ AGTACCTGAGGCCGGTCTCAGTA</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Probe – 5’ ACTGGGTGGGATGTCTTTC</td>
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### Supplemental Table 1B. qPCR primer information: SYBR Green Probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Purpose</th>
<th>Forward Primer sequence</th>
<th>Reverse Primer sequence</th>
<th>Gene ID</th>
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<tbody>
<tr>
<td>18S ribosomal RNA (18S)</td>
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<td>5’-ACGGCTACCACATCCAGGA</td>
<td>5’-CCAATTACAGGCCTGAAA</td>
<td>NR_003286</td>
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<tr>
<td>Beta-actin (ACTB)</td>
<td>Reference gene</td>
<td>5’-CAAAAGCCACCCGTTTCT</td>
<td>5’-TTCTCTTCCCTCCCTGTGT</td>
<td>XM_536888</td>
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Supplemental Table 2. Statistical significance of candidate miR plasma-concentration differences between controls and patients with AF only, CHF only and AF along with CHF, controlling for a variety of potential contributors

<table>
<thead>
<tr>
<th>miR29b (x10^5)</th>
<th>AF</th>
<th>CHF</th>
<th>AF + CHF</th>
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</thead>
<tbody>
<tr>
<td>Beta (95% CI)</td>
<td>P</td>
<td>Beta (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Model 1*</td>
<td>-11.8 (-18.6 - 5.0)</td>
<td>0.002</td>
<td>-11.4 (-16.6 - -6.2)</td>
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<tr>
<td>Model 2*</td>
<td>-11.3 (-18.3 - -4.3)</td>
<td>0.002</td>
<td>-13.0 (-19.8 - -6.2)</td>
</tr>
<tr>
<td>Model 3*</td>
<td>-10.5 (-17.9 - -3.1)</td>
<td>0.007</td>
<td>-10.4 (-20.6 - -3.2)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>miR21 (x10^5)</th>
<th>AF</th>
<th>CHF</th>
<th>AF + CHF</th>
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<tbody>
<tr>
<td>Beta (95% CI)</td>
<td>P</td>
<td>Beta (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Model 1</td>
<td>-2.7 (-6.0 - 0.71)</td>
<td>0.20</td>
<td>-2.3 (-4.9 - 0.3)</td>
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<tr>
<td>Model 2</td>
<td>-3.5 (-6.9 - -0.0)</td>
<td>0.05</td>
<td>-3.0 (-6.3 - 0.3)</td>
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<tr>
<td>Model 3</td>
<td>-3.7 (-7.3 - -0.2)</td>
<td>0.041</td>
<td>-3.9 (-8.7 - 1.2)</td>
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</table>

<table>
<thead>
<tr>
<th>miR133b (x1)</th>
<th>AF</th>
<th>CHF</th>
<th>AF + CHF</th>
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</thead>
<tbody>
<tr>
<td>Beta (95% CI)</td>
<td>P</td>
<td>Beta (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Model 1</td>
<td>-0.86 (-1.76 - 0.03)</td>
<td>0.06</td>
<td>-1.13 (-1.81 - -0.44)</td>
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<tr>
<td>Model 2</td>
<td>-0.91 (-1.87 - 0.04)</td>
<td>0.06</td>
<td>-1.12 (-2.03 - -0.21)</td>
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<tr>
<td>Model 3</td>
<td>-0.86 (-1.88 - 0.15)</td>
<td>0.09</td>
<td>-1.04 (-2.40 - 0.32)</td>
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</table>

<table>
<thead>
<tr>
<th>miR15 (x10^3)</th>
<th>AF</th>
<th>CHF</th>
<th>AF + CHF</th>
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<tr>
<td>Beta (95% CI)</td>
<td>P</td>
<td>Beta (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Model 1</td>
<td>1.1 (-1.9 - 4.0)</td>
<td>0.46</td>
<td>-0.63 (-1.63 - 0.38)</td>
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<tr>
<td>Model 2</td>
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<tr>
<td>Model 3</td>
<td>1.9 (-1.3 - 5.1)</td>
<td>0.23</td>
<td>-0.73 (-2.69 - 1.23)</td>
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</tbody>
</table>

*Model 1 adjusted for age and sex only. Model 2 adjusted for coronary artery disease, hypertension, diabetes mellitus, and hypercholesterolemia in addition to age and sex. Model 3 adjusted for use of angiotensin converting enzyme inhibitor or AT1-receptor blocker, aldosterone antagonists, and/or statins in addition to the factors included in model 2.
Supplemental Table 3. Virus genome copy number content of 200 μL single bolus injections and the number of animals treated with each virus batches

<table>
<thead>
<tr>
<th></th>
<th>GFP-control</th>
<th>Mir29b-sponge</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3.8 x 10^{11}, n=3</td>
<td>5.8 x 10^{11}, n=6</td>
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<tr>
<td></td>
<td>8.9 x 10^{11}, n=3</td>
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<td></td>
<td>3.9 x 10^{11}, n=2</td>
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</tr>
<tr>
<td>Average</td>
<td>5.7 x 10^{11}</td>
<td>4.8 x 10^{11}</td>
</tr>
</tbody>
</table>
Supplemental Figure 1

Masson Trichrome-stained images of LA tissue from representative control (CTL), a 24 hr ventricular-tachypaced (VTP), 1 wk VTP and 2 wk VTP dogs. Images are taken at x200 magnification and the scale bar represents 200 µm.
Left-atrial miR29b expression in 5 sham dogs (instrumented like CHF dogs but with pacemaker deactivated and followed for 1 week) versus control (CTL) dogs.
Supplemental Figure 3

Expression of miR29a (A) and miR29c (B) in freshly-isolated fibroblasts from LA of control (CTL) dogs and dogs subjected to VTP for the durations indicated (Ns are numbers of dogs/group; *P<0.05, **P<0.01, ***P<0.001 vs CTL.)
Expression of miR590 (A) miR133a (B) and miR133b (C) in freshly-isolated fibroblasts from LA of control (CTL) dogs and dogs subjected to VTP for the durations indicated (Ns are numbers of dogs/group; *P<0.05, **P<0.01, ***P<0.001 vs CTL)
Expression of GFP (A), in control (SCR, top panel) and miR29b-sponge knockdown (bottom panel) conditions, in ventricular tissues. Corresponding bright-field images (B) for control mice (top panel) and miR29b-sponge knockdown (bottom panel). Green is GFP and blue are propidium iodide-stained nuclei. Horizontal bar= 20 µm.
**Supplemental Figure Legends**

**Supplemental Figure 1.** Representative left atrial tissue sections stained with Masson Trichrome to assess fibrous tissue content. Representative images are shown from CTL dogs and VTP-dogs at each VTP time point: 24 hour (hr) VTP, 1 week (wk) VTP, and 2 wk VTP.

**Supplemental Figure 2.** MiR-29b expression was compared in LA tissue from sham versus CTL dogs. Samples were subjected to qPCR in parallel at the same time for optimal comparability. Sham dogs underwent the same surgery and instrumentation as VTP dogs but their pacemaker was not activated. Sham dogs were followed for 1 week and then sacrificed. \( P=0.36 \) by unpaired t-test (n=5/group).

**Supplemental Figure 3.** MiR-29a and miR-29c expression time-course as function of VTP-duration. (A) Changes in miR29a-expression in freshly isolated LA fibroblasts, (B) changes in miR29c-expression in freshly isolated LA fibroblasts. \( \ast P<0.05, \ast\ast P<0.01, \ast\ast\ast P<0.001 \) vs. CTL by one-way ANOVA followed by a Dunnett’s post-hoc test. Numbers indicate number of dogs/group. All data are presented as mean±SEM.

**Supplemental Figure 4.** The expression of miR-590 (A), miR-133a (B) and miR-133b (C) was measured in freshly isolated atrial fibroblasts from CTL and VTP
time-course dogs. (*P<0.05, **P<0.01, ***P<0.001 vs. CTL by one-way ANOVA followed by a Dunnett’s post-hoc test. Numbers indicate number of dogs/group. All data are presented as mean±SEM).

**Supplemental Figure 5.** Expression of GFP (A), in control (SCR, top panel) and miR29b-sponge knockdown (bottom panel) conditions, in ventricular tissues. Corresponding bright-field images (B) for control mice (top panel) and miR29b-sponge knockdown (bottom panel). Green is GFP and blue are propidium iodide-stained nuclei. Horizontal bar= 20 µm.