Role for MicroRNA-21 in Atrial Profibrillatory Fibrotic Remodeling Associated With Experimental Postinfarction Heart Failure

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Background—Atrial tissue fibrosis is often an important component of the atrial fibrillation (AF) substrate. Small noncoding microRNAs are important mediators in many cardiac remodeling paradigms. MicroRNA-21 (miR-21) has been suggested to be important in ventricular fibrotic remodeling by downregulating Sprouty-1, a protein that suppresses fibroblast proliferation. The present study examined the potential role of miR-21 in the atrial AF substrate resulting from experimental heart failure after myocardial infarction (MI).

Methods and Results—Large MIs (based on echocardiographic left ventricular wall motion score index) were created by left anterior descending coronary artery ligation in rats. Changes induced by MI versus sham controls were first characterized with echocardiography, histology, biochemistry, and in vivo electrophysiology. Additional MI rats were then randomized to receive anti–miR-21 (KD21) or scrambled control sequence (Scr21) injections into the left atrial myocardium. Progressive left ventricular enlargement, hypocontractility, left atrial dilation, fibrosis, refractoriness prolongation, and AF promotion occurred in MI rats versus sham controls. Atrial tissues of MI rats showed upregulation of miR-21, along with dysregulation of the target genes Sprouty-1, collagen-1, and collagen-3. KD21 treatment reduced atrial miR-21 expression levels in MI rats to values in sham rats, decreased AF duration from 417 (69–1595; median [Q1–Q3]) seconds to 3 (2–16) seconds (8 weeks after MI; P<0.05), and reduced atrial fibrous tissue content from 14.4±1.8% (mean±SEM) to 4.9±1.2% (8 weeks after MI; P<0.05) versus Scr21 controls.

Conclusions—MI-induced heart failure leads to AF-promoting atrial remodeling in rats. Atrial miR-21 knockdown suppresses atrial fibrosis and AF promotion, implicating miR-21 as an important signaling molecule for the AF substrate and pointing to miR-21 as a potential target for molecular interventions designed to prevent AF. (Circ Arrhythm Electrophysiol. 2012;5:1027-1035.)

Key Words: antiarrhythmia agents • arrhythmia • electrophysiology • fibrillation • remodeling

Atrial fibrillation (AF) affected 2.2 million Americans and 4.5 million Europeans in 2006.1 Several cardiovascular conditions are known to cause AF, including cardiomyopathies,2 mitral valve disease,3 myocarditis,4 and hypertension.5 Heart failure (HF), one of the most common causes of AF,6 produces atrial electric and structural alterations, with atrial tissue fibrosis being a major component.8 In a canine model of ventricular tachypacing-induced HF, progressive atrial fibrosis is associated with the AF substrate.7 Recovery from HF reverses the hemodynamic and electrophysiological consequences, but AF promotion and atrial fibrosis remain.9 Angiotensin-converting enzyme inhibitors suppress fibrosis and AF promotion.7 Renin-angiotensin system inhibitors may protect patients with HF from AF.10

Fibroblasts secrete extracellular matrix components and regulators, such as collagen, fibronectin, matrix metalloproteases, and tissue inhibitors of metalloproteinases.11 HF fibroblasts proliferate and differentiate to become myofibroblasts under the influence of paracrine and autocrine factors.11,12 Myofibroblast activation increases production of collagen, fibronectin, and cytokines that regulate extracellular matrix production and breakdown, leading to extracellular matrix accumulation and fibrosis.12

Recently, small endogenous noncoding RNAs, called microRNAs (miRs), have been shown to bind 3′-untranslated regions of target mRNAs to alter protein translation and mRNA stability13 and to participate in ventricular remodeling.14,15 Thum et al16 showed that miR-21 is upregulated in ventricular fibroblasts of mice subjected to transverse aortic constriction.

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and that miR-21 knockdown suppresses adverse ventricular remodeling. MiR-21 targets Sprouty-1, a negative regulator of extracellular signal regulated kinases (ERK1/2), which enhance fibroblast survival and density.\textsuperscript{16} Thus, miR-21 is a candidate to participate in AF-promoting atrial fibrotic remodeling. It is important to clarify unresolved mechanisms underlying the AF substrate to design novel approaches to clinical management.\textsuperscript{17} The present study was designed (1) to assess the changes in atrial tissue properties, arrhythmia vulnerability, and structure, along with miR-21 and its target genes, in HF caused by extensive myocardial infarction (MI) in rats, and (2) to determine whether miR-21 knockdown attenuates atrial fibrosis and AF promotion in this model.

**Materials and Methods**

For additional details, see the online-only Data Supplement.

**Animals**

Male Wistar rats, weighing 200 to 250 g (Charles River, Saint-Constant, Quebec, Canada), were fed rat chow and housed under a 12:12-hour light/dark cycle at 21°C and 50% humidity. Rats had free access to tap water and food. Animal handling procedures followed the guidelines of the National Institutes of Health and were approved by the Montreal Heart Institute Animal Ethics Committee.

**Surgical Procedures**

Rats were anesthetized with 3% isoflurane, intubated, and ventilated after preoperative buprenorphine (0.03 mg/kg SC) injection. The thorax was shaved and sterilized with 2% w/v chlorhexidine gluconate in 70% v/v isopropyl alcohol. A left thoracotomy was performed, and the left anterior descending coronary artery was ligated with 6-0 silk. MI rats were compared with sham controls that underwent similar procedures but without left anterior descending ligation. The chest was closed with 3-0 silk and the skin closed with autoclips. Additional doses of buprenorphine (0.03 mg/kg) were administered subcutaneously 6 and 12 hours postoperatively.

**Transthoracic Echocardiography**

Transthoracic echocardiographic studies under 3% isoflurane were obtained before and 2 weeks and 8 weeks after surgery with a phased-array 10S probe (4.5–11.5 MHz) in a Vivid 7 Dimension System (GE Healthcare Ultrasound, Horten, Norway).

Echocardiographic studies were used to select rats with large MIs. Left ventricular (LV) regional wall motion was scored in LV short axis for the 6 segments in this view as follows: (1) normal, (2) hypo-kinesia, (3) akinesia, (4) dyskinesia, and (5) aneurysmal. Wall motion score index (WMSI) was the mean value of all scores. Infarct size was classified as follows: small (<WMSI<1.25); medium (1.25<WMSI<1.65); or large (WMSI≥1.65). MI rats with WMSI<1.65 2 weeks after MI were excluded from the study.

Further analysis included LV dimensions (LV areas at end diastole and systole), systolic function (LV fractional area change), diastolic function (the mitral-valve inflow E-wave to early-diastolic mitral-valve annulus velocity ratio [E/Em ratio]), and myocardial performance index. Atrial analyses included left atrial (LA) dimension at end diastole and systole, right atrial dimension, and LA fractional shortening. Additional echocardiographic analyses and results are detailed in the online-only Data Supplement.

**Hemodynamic Measurements**

Systemic and intraventricular hemodynamic parameters were measured with 2.0F and 3.0F Millar catheters inserted into the right carotid artery, left ventricle (via right carotid artery), and right ventricle (via right jugular vein) in animals anesthetized with 2.5% to 3.0% isoflurane.

**In Vivo Electrophysiology**

Rats were intubated and ventilated under 3% isoflurane anesthesia. An octopolar catheter (1.9F) with 0.5-mm interelectrode spacing (Scisense) was inserted into the right atrium via jugular vein for stimulation and recording. A custom-built computer-based stimulator was used to trigger a stimulus isolator (Grass Telefactor model SD9K) with selected stimulation protocols. Atrial effective refractory period was determined (as the longest S1-S2 interval that failed to generate a response) with twice-threshold, 2-ms, square-wave pulses, 15-stimulus drive trains (S1) at 100-ms cycle length, followed by an S2 decremented in 2-ms intervals. We recorded surface ECG, intracardiac electrograms, and stimulus artifacts with IOX software (EMKA technologies, Paris, France) for monitoring and off-line analysis. In each animal, atrial effective refractory periods, Wenckebach cycle length, and corrected sinus node recovery time were determined with twice-threshold, 2-ms, square-wave pulses. AF was induced with up to 3 extrastimuli at a cycle length of 100 ms and, if that failed, atrial burst pacing. A standardized protocol was used (see the online-only Data Supplement for additional details). Rapid and irregular atrial rate (>500 beats per minute) with varying electrogram morphology characterized AF. The maximum AF duration in each animal was used to reflect the AF substrate. At the end of experiments, hearts were excised and immersed into buffered formalin or snap frozen in liquid N\textsubscript{2} for further analysis.

**Histology**

Hearts were preserved in 10% buffered formalin for paraffin embedment. Serial atrial tissue sections (12-μm thickness, 500-μm spacing) were stained with Masson Trichrome. Analysis of ×400 magnification images (15–20 from 3–5 sections) was performed with Image-Pro 6.2 software. Perivascular areas were avoided. Fibrous tissue content was expressed as a percentage of field area and averaged across fields for each rat.

**Protein Extraction and Immunoblots**

Protein extracts (100 μg) of snap-frozen LA tissue were separated by electrophoresis on 12% SDS-PAGE and transferred to nitrocellulose membranes (0.45 A). Membranes were blocked and incubated overnight with the primary antibodies and then with secondary antibodies. Antibody signals were visualized with enhanced chemiluminescence. Data were normalized to GAPDH band intensity from the same samples on the same membranes. The following primary antibodies were used: mouse anti–phospho-P38 mitogen-activated protein (MAP) kinase, mouse anti–phospho-ERK1/2 MAP kinase, mouse anti-P38 MAP kinase, mouse anti-ERK1/2 MAP kinase (1:2000; Cell Signaling Technology, Danvers, Mass), and mouse anti-GAPDH (1:10 000; RDI, Fitzgerald Industries). Secondary antibody was horseradish peroxidase–conjugated anti-mouse (1:10 000; Jackson ImmunoResearch Laboratories). For further details, see the online-only Data Supplement.

**Quantitative Real-Time Polymerase Chain Reaction Analysis**

RNA was isolated with mirVana isolation kits (Ambion/Life Technology, Carlsbad, CA). Real-time quantitative polymerase chain reaction for miR-21 was performed with carboxy-fluorescein (FAM)-labeled fluorescent TaqMan assay primers (Applied Biosystems, Foster City, CA) and TaqMan Universal Master Mix (Applied Biosystems). Relative quantifications were calculated with the comparative threshold cycle method (2−ΔΔC\textsubscript{T}), with snuU6 as an internal standard. Quantitative polymerase chain reaction for Sprouty-1, procollagen Ia, and procollagen IIIa was performed with miR-21 as an internal standard. Quantitative polymerase chain reaction for Sprouty-1, procollagen Ia, and procollagen IIIa was performed with miR-21 as an internal standard.
In Vivo MiR-21 Knockdown

Initial model characterization pointed to a role of miR-21 in adverse remodeling. Therefore, experiments were performed in 4 additional groups of rats: (1) sham-operated rats with a miR-21 knockdown probe anti–miR-21 (KD21; online-only Data Supplement Figure SI) injected into LA myocardium; (2) sham-operated rats injected with scrambled control probe (Scr21); (3) MI rats injected with KD21; and (4) MI rats injected with Scr21. The KD21 probe was a locked nucleic acid 15-mer oligonucleotide (TCAGTCTGATAAGCT), with a complete phosphorothioate backbone purified by high-performance liquid chromatography. The Scr21 probe (CGTCTAGCCACCTAG), predicted bioinformatically not to interact with any other gene, was a negative control. Both constructs were custom synthesized with locked nucleotide chemistry by Exiqon (Vedbaek, Denmark). KD21 and Scr21 were dissolved in normal saline solution at 5 μg/μL. After thoracotomy, 10-μL solution was injected with a Hamilton microsyringe (31-gauge needle) into the LA wall at 5 separate injection points. In MI rats, left anterior descending ligation was performed after completing LA myocardial injections. In sham rats, LA myocardial injections were performed without left anterior descending ligation. After 2- or 8-week periods following MI, rats were submitted to echocardiography, in vivo electrophysiological study, and cardiac excision for histology or quantitative polymerase chain reaction.

Statistical Analysis

Variables fulfilling criteria for normal distribution (Shapiro-Wilks test) are expressed as mean±SEM; those that do not are expressed as median and first and third quartile (Q1–Q3) values delimiting both test) are expressed as mean±SEM; those that do not are expressed as median and first and third quartile (Q1–Q3) values delimiting bot-

Results

Characterization of the Model

To characterize model evolution, serial echocardiograms were obtained at preoperative baseline, and 2 weeks and 8 weeks after MI. Key findings are shown in Figure 1A to 1E, and results of additional analyses are shown in the online-only Data Supplement Figure SII. Clear and important atrial remodeling occurred. LA dimensions increased progressively, and atrial fractional shortening decreased (Figure 1A–1C). Significant right atrial dilation also occurred 8 weeks after MI (online-only Data Supplement Figure SIIA). LV dimensions increased after MI (online-only Data Supplement Figure SIIIB and SIIC), whereas LV fractional area change deteriorated progressively (Figure 1D), indicating substantial LV systolic dysfunction. Global myocardial performance index worsened progressively (online-only Data Supplement Figure SIID). The WMSI increased substantially after MI (online-only Data Supplement Figure SIIIE). Hemodynamic measurements (Table 1) showed increased LV filling pressures and reduced pressure generation rates.

Representative Masson Trichrome images at 1, 2, 4, and 8 weeks after surgery in sham and MI rats are shown in Figure 1F. Fibrous tissue content increased significantly at 2 weeks after MI and remained elevated thereafter (Figure 1G).

Electrophysiology

Atrial effective refractory periods increased significantly after MI (P<0.001 for group effect; Table 2), with no time-related evolution during 2 to 8 weeks. Corrected sinus node recovery time was unaffected. Wenckebach cycle length also increased significantly after MI. Maximum AF duration per rat increased significantly 8 weeks after MI (online-only Data Supplement Figure SIII and Table 2).

Biochemistry

MAP kinase phosphorylation changes have been implicated in atrial fibrotic remodeling.7,8 To obtain mechanistic insights
into atrial remodeling of HF rats after MI, we studied the changes in P38 and ERK1/2 expression and phosphorylation. Original Western blots from 1-week after MI and corresponding sham rats are shown in Figure 2A, with corresponding temporal evolution data in Figure 2B to 2E. ERK1/2 phosphorylation increased 1 week after MI and returned to control values after 4 weeks. Total ERK1/2 expression also increased, but to a lesser extent than phospho-ERK1/2, 1 week after MI. P38 expression and phosphorylation were unchanged.

Because miR-21 has been implicated in ventricular fibrosis after MI, acting via Sprouty-1 to cause ERK hyperphosphorylation,16 we suspected the involvement of miR-21. Therefore, we measured the expression of miR-21, as well as that of its primary target Sprouty-1, in MI rats and corresponding shams at the time of steady-state fibrosis, 2 weeks after MI. MiR-21 expression was increased 2.5-fold in MI rats (Figure 3A), Sprouty-1 was strongly downregulated (Figure 3B), and procollagen I and procollagen III were upregulated (Figure 3C), consistent with an important role of miR-21.

To assess more critically the participation of miR-21, we designed additional experiments to knock down LA miR-21 by injecting anti–miR-21 (KD21) into rat LA tissue at the time of coronary artery ligation, comparing the findings with KD21-injected contemporary sham rats, as well as sham and MI rats receiving Scr21 LA injections.

We first confirmed effective miR-21 knockdown (Figure 4A). LA miR-21 expression was increased at 2 weeks in Scr21 rats subjected to MI surgery in comparison with sham, indicating that injection of the scrambled sequence did not prevent miR-21 upregulation after MI. By 8 weeks after MI, there were no longer statistically significant miR-21 differences between MI and sham rats, consistent with the lack of fibrosis progression between weeks 2 and 8 (Figure 1G). KD-21 significantly reduced maximal AF duration 8 weeks after MI to values comparable with those of sham rats (Figure 4C). Figure 5A shows representative Masson Trichrome images of LA samples from rats 8 weeks after surgery. Scr21-injected rats had substantial increases

Table 1. Hemodynamic Measurements

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<th></th>
<th>1 wk</th>
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<th>2 wk</th>
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<th>8 wk</th>
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<td></td>
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<td>MI (n=9)</td>
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<td>90±5</td>
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<td>−2±1</td>
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<td>−1±1</td>
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<td>12±2</td>
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<td>3±1</td>
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<td>4±1</td>
<td>21±1</td>
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<td>dP/dt max, mm Hg/s †</td>
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<td>3912±290</td>
<td>5296±220</td>
<td>4258±325</td>
<td>5145±318</td>
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<td>dP/dt min, mm Hg/s †</td>
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<td>RV pressures</td>
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<td>25±2</td>
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<td>−3±1</td>
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<td>5±1</td>
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<td>−1301±14</td>
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</tbody>
</table>

MI indicates myocardial infarction; LV, left ventricular; RV, right ventricle. Results are mean±SEM.

*P<0.05, †P<0.001, sham vs MI (group main effect).

Table 2. Electrophysiological Parameters

<table>
<thead>
<tr>
<th></th>
<th>2 wk</th>
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<th>4 wk</th>
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<th>8 wk</th>
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<tbody>
<tr>
<td></td>
<td>Sham (n=5)</td>
<td>MI (n=12)</td>
<td>Sham (n=12)</td>
<td>MI (n=15)</td>
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<td>AERP100, ms</td>
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<td>SNRTc100, ms</td>
<td>39±2</td>
<td>42±12</td>
<td>40±11</td>
<td>38±15</td>
<td>36±4</td>
<td>31±6</td>
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<tr>
<td>SNRTc80, ms</td>
<td>30±10</td>
<td>31±6</td>
<td>35±9</td>
<td>17±5</td>
<td>35±7</td>
<td>25±6</td>
</tr>
<tr>
<td>WCL, ms*</td>
<td>129±9</td>
<td>156±19</td>
<td>144±7</td>
<td>151±19</td>
<td>137±4</td>
<td>161±17</td>
</tr>
<tr>
<td>Maximum AF duration, s, median (Q1–Q3)</td>
<td>2.8 (1.7–10.7)</td>
<td>5.4 (1.3–78.2)</td>
<td>4.1 (1.8–189.8)</td>
<td>1.9 (1.4–78.2)</td>
<td>1.8 (1.4–5.6)</td>
<td>152.6 (47.1–1029)</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction; AERP, atrial effective refractory period; SNRTc, corrected sinus node recovery time; WCL, Wenckebach cycle length; AF, atrial fibrillation. Results are mean±SEM unless otherwise indicated.

*P<0.001, sham vs MI (group main effect); †P<0.01, MI 8 wk vs sham 8 wk (Kruskal-Wallis analysis with Dunn post hoc test).
in LA fibrous tissue content versus matched shams. KD21 suppressed this fibrotic response, reducing fibrous tissue content by \( \approx 66\% \) versus Scr21-injected MI rats (Figure 5B). We also studied P-wave duration as an index of atrial conduction because atrial fibrosis promotes AF maintenance by affecting conduction.\(^7,9,18\) KD21 significantly decreased P-wave duration (Figure 5C).

Intra-atrial KD21 administration did not significantly mod-
ify MI-induced increases in atrial effective refractory period and Wenckebach cycle length, nor did it affect corrected sinus node recovery time (Figure 6). PR intervals, QRS durations, and QT intervals increased in MI rats, with no effect of KD21 administration, supporting atrial-predominant actions consistent with LA-directed KD21 administration (Figure 7).

To further assess cardiac remodeling, we performed echo-
cardiography in KD21 and Scr21-injected rats (online-only Data Supplement Figure SIV). Similar to the basic characterization shown in Figure 1A and 1B, LA dimensions increased within 2 weeks in MI rats; these changes were unaffected by KD21. Similarly, LV function worsened after MI but was unaffected by KD21. These results indicate that the beneficial effects of miR-21 knockdown on the atrial arrhythmogenic substrate were not secondary to changes in ventricular function or the severity of HF, that LA dilation is not because of atrial fibrosis, and that AF substrate development can be prevented without attenuating LA enlargement.

### Discussion

In this study, we characterized atrial profibrillatory remodeling in rats with MI-induced HF, observing changes compatible with profibrotic signaling via miR-21. We then tested the role of miR-21 directly, with atrial knockdown of miR-21. We found that miR-21 knockdown suppressed LA fibrotic remodeling, reducing tissue fibrosis and AF persistence significantly in rats with HF after MI.

### Comparison With Previous Observations of Cardiac Remodeling in Related Models

MIs commonly induce LV dysfunction and HF in humans, causing a range of comorbidities, including AF.\(^19\) Ventricular remodeling in rats roughly parallels corresponding phenomena in humans.\(^20\) Milliez et al\(^21\) reported LA enlargement associated with atrial fibrosis and ectopy that correlated with LV dysfunction in rats 3 months after MI. Boixel et al\(^22\) noted that atrial fibrosis in rats after MI is associated with important hemodynamic disturbances and accumulation of matrix metalloproteinase-7. Atrial dilation and fibrosis are also prominent features in other experimental HF paradigms, such as ovine and canine ventricular tachycardiomypathy.\(^23-26\) Both direct interference with cardiomyocyte bundle continuity and arrhythmogenic electrical interactions between cardiomyocytes and fibroblasts might contribute to AF promotion by atrial fibrosis.\(^27\)

### MicroRNAs and AF

MicroRNAs are important in a wide range of cardiac remodeling processes,\(^28\) and there is increasing evidence for participation in AF.\(^13\) \(I_{K1}\) upregulation contributes to AF stabilization because of electrical remodeling,\(^29\) and miR-1 has been implicated,\(^30\) although there is also evidence for a role of miR-26.\(^13\) \(I_{Ca\text{L}}\) downregulation is central to AF-related action potential abbreviation and AF promotion,\(^29\) to which miR-328 upregulation may contribute.\(^31\)

It is also likely that miRs are important in atrial structural remodeling, particularly fibrosis. Several miRs may mediate cardiac fibrosis, with the most likely candidates being miR-21, miR-29, miR-133, miR-30, and miR-590.\(^32\) MiR-133 and miR-590 may participate in atrial fibrotic remodeling caused by chronic nicotine exposure.\(^33\) Here, we found that miR-21 is upregulated in the atrial profibrotic milieu caused by MI-associated HF and showed that suppression of miR-21 upregulation suppresses both atrial fibrosis and AF promotion.
MiR-21 and Cardiac Remodeling

MiR-21 is involved in fibrotic remodeling of the lungs and kidneys. Thum et al. showed upregulation of miR-21 in a transverse aortic constriction model of HF. They also identified Sprouty-1 as a target of miR-21 and demonstrated Sprouty-1 repression by miR-21 in transfected cardiac fibroblasts. MiR-21 upregulation was associated with downregulation of Sprouty-1 and ERK hyperphosphorylation. Knockdown of cardiac miR-21 by tail-vein injection of miR-21 antagonist suppressed cardiac ERK phosphorylation, reduced interstitial fibrosis, and attenuated ventricular dysfunction. The importance of miR-21 in ventricular remodeling was disputed by Patrick et al., who showed that miR-21 knockout and inhibition by 8-nucleotide anti-miRs failed to prevent ventricular hypertrophic and fibrotic responses in mice subjected to pressure overload. In response, Thum et al. argued that miR-21 knockout from birth may be followed by adaptive responses, well recognized in knockout models, and showed that 8-nucleotide anti-miRs produce only modest and transient changes in miR-21 expression, resulting in lack of efficacy. Here, we used 15-nucleotide–locked nucleic acid–modified in vivo knockdown anti-miRs, which effectively prevented MI-induced upregulation of miR-21.

In the present study, we elected to use direct atrial myocardial injection of miR-21 anti-miR to avoid indirect atrial protection mediated by improved ventricular function as might occur after systemic anti-mir injection. Thum et al. noted improved LV function in transverse aortic constriction mice treated with systemic miR-21 antagonist injection. Indeed, ventricular dysfunction was unchanged in our KD21 rats compared with Scr21 controls (online-only Data Supplement Figure SIV), excluding indirect effects secondary to improved hemodynamics and indicating direct participation of atrial miR-21 upregulation in AF-promoting atrial structural remodeling.

Novel Elements and Consideration of the Model

The present study is the first of which we are aware to directly demonstrate the role of an miR in AF-promoting atrial fibrotic remodeling. Furthermore, they constitute a proof of principle of the potential beneficial consequences of suppressing miR-21 upregulation in the prevention of AF.
Interestingly, AF promotion was suppressed with reduced atrial fibrosis, despite persistent LA dilation. These findings are consistent with previous work in which AF promotion persisted along with continued atrial fibrosis, although LA dilation was reversed after hemodynamic recovery from HF. These results suggest that atrial fibrosis is an essential factor in the HF-related AF substrate, providing support for upstream therapy that prevents or reverses atrial fibrosis in AF management.

Although systemic anti–miR-21 administration might protect against both HF-induced atrial structural remodeling and LV function impairment, side effects of systemic administration might be a clinical problem. For example, anti-apoptotic effects of miR-21 are needed for ischemic preconditioning and prevent abdominal aortic aneurysm dilation. Local administration, though technically challenging, could overcome these barriers and provide direct, tissue-specific miR-21 inhibition.

We chose a rat HF model because reagent costs for miR-21 knockdown in larger animals like dogs or pigs would have been prohibitive. The ventricular tachycardiomypathy model was unavailable because of a lack of implantable pacemakers appropriate for rats, so we employed a widely used MI model to induce HF. An advantage of the model of HF after MI is its potential relevance to HF in patients with prior MI, a common cause of clinical HF. To standardize infarct size and study animals with significant HF, we required large MIs based on echocardiographic WMSI for inclusion in the study. This decision had the advantage of creating a relatively uniform pool of animals: ventricular and atrial echocardiographic indices were well matched between KD21 and Scr21 rats (online-only Data Supplement Figure SIV). The major disadvantage was a high drop-out rate: only 1/3 of rats had sufficiently large MIs. Combined with a high early mortality rate in rats with large MI (≈50% within 24 hours), this approach greatly limited the numbers of animals we could study.

Because of species- and disease-specific considerations, our results should not be extrapolated directly to other models of atrial fibrosis/AF. Further investigation will be needed to assess their applicability to clinical AF and other experimental HF paradigms. Although our data strongly support a significant role of miR-21 in atrial fibrotic remodeling in MI-related HF, they do not exclude contributions from other miRs, which remain to be addressed in future work.

Our results show that the inhibition of atrial fibrosis by suppression of miR-21 upregulation coincides with prevention of AF substrate formation in this rat model of HF due to MI. These findings do not prove that fibrosis mitigation mediated the anti-AF effects. For example, benefit may have been because of the prevention of fibroblast proliferation and fibroblast-cardiomyocyte electrical interaction. Similarly, additional atrial protective effects or remote nonatrial actions cannot be completely excluded based on our results. That fibrosis alone is insufficient to sustain AF in our model is indicated by the fact that fibrosis reached a maximum 2 weeks after MI (Figure 1G) but AF duration did not increase significantly until 8 weeks after MI (Figure 4C and online-only Data Supplement Figure SIII). The echocardiographic data showing progressive LA dilation between weeks 2 and 8 point to LA enlargement as a factor that may also have been required for AF substrate development. The simplest explanation of our results is that both fibrosis and LA enlargement are needed for the AF substrate; thus, at 2 weeks (when there is fibrosis but insufficient LA enlargement) or 8 weeks after MI+KD21 administration (when insufficient fibrosis has developed despite substantial LA enlargement) prolonged AF does not occur.

Conclusions

Atrial fibrosis and AF promotion in rats with HF after MI are associated with miR-21 and target gene expression changes consistent with miR-21 signaling. Suppression of MI-induced...
miR-21 upregulation prevents atrial fibrosis and AF promotion. These findings implicate miR-21 as a significant signaling molecule in atrial proarrhythmic remodeling and establish it as a potentially interesting target for AF-preventing therapy.

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Disclosures
Dr Nattel is listed as the inventor in a patent belonging to the Montreal Heart Institute and Université de Montréal, entitled “miR-21 as a target in prevention of atrial fibrillation.” The other authors have no conflicts to report.

References


**CLINICAL PERSPECTIVE**

Better insights into underlying mechanisms are needed to improve our understanding of the pathophysiology of atrial fibrillation (AF) and to devise innovative treatment approaches. Atrial tissue fibrosis is believed to be important in many forms of AF. MicroRNAs (miRs) are short RNA sequences that regulate gene expression by inhibiting protein translation and destabilizing mRNA. MiR-21 governs that fibroblast survival plays a role in a variety of fibrotic processes. We assessed the involvement of miR-21 in a rat model of fibrosis-related AF. Rats subjected to a large acute anterior myocardial infarction were compared with sham-operated rats. Myocardial infarction rats developed severe left ventricular dysfunction and left atrial enlargement, were susceptible to prolonged AF in response to atrial burst pacing, manifested 2.5-fold increases in miR-21 expression, and showed other gene/protein expression changes compatible with miR-21 signaling. Knocking down atrial miR-21 expression with an anti-miR molecule injected into the left atrium at the time of initial surgery attenuated atrial fibrosis and prevented prolonged AF. These results implicate miR-21 as an important signaling molecule in AF-promoting fibrosis and point to its potential value as a novel therapeutic target for AF prevention.
Role for MicroRNA-21 in Atrial Profibrillatory Fibrotic Remodeling Associated With Experimental Postinfarction Heart Failure
Sophie Cardin, Eduard Guasch, Xiaobin Luo, Patrice Naud, Khaï Le Quang, YanFen Shi, Jean-Claude Tardif, Philippe Comtois and Stanley Nattel

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SUPPLEMENTAL MATERIALS

A Role for MicroRNA-21 in Atrial Profibrillatory Fibrotic Remodeling Associated with Experimental Post-infarction Heart Failure

Sophie Cardin, PhD*; Eduard Guasch, MD*; Xiaobin Luo, MSc; Patrice Naud, PhD; Khaï Le Quang, MD, PhD; YanFen Shi, PhD; Jean-Claude Tardif, MD; Philippe Comtois, PhD; Stanley Nattel, MD

SUPPLEMENTARY METHODS

Transthoracic Echocardiography

Transthoracic echocardiographic studies (under 3%-isoflurane) were obtained before, 2 weeks, and 8 weeks after surgery, with a phased-array 10S probe (4.5 - 11.5 Megahertz) in a Vivid 7 Dimension system (GE Healthcare Ultrasound, Horten, Norway).

**MI-Model:** A left-ventricular (LV) short-axis view at the level of the papillary muscles was recorded to define MI-area. LV regional wall motion was scored as: normal 1, hypokinesis 2, akinesis 3, dyskinesis 4, and aneurysmal 5, for the 6 segments in this view. Wall-motion score index (WMSI) was the mean-value of all scores. Infarct-size was classified as: small (1<WMSI≤1.25); medium (1.25<WMSI<1.65) or large (WMSI≥1.65). MI-rats with WMSI<1.65 2 weeks post-MI were excluded from the study.
LV Structural and Functional Remodeling: A) Systolic function: LV-areas at end-diastole (LVAd) and systole (LVAs) were measured in short-axis view. LV fractional area-change (LV-FAC) was calculated as (LVAd–LVAs)/LVAd×100%. LV M-mode in this view was used to measure LV-dimension at end-diastole (LVDd) and systole (LVDs). B) Diastolic function: Pulsed-wave Doppler was used to study transmitral flow (TMF) in apical 4-chamber view and peak velocity in early-filling E wave. Mitral annulus velocity during early filling (Em) was measured by tissue-Doppler. E/Em ratio was calculated at the lateral annulus. C) Myocardial performance: The time-interval from mitral closing to opening (MVco) was measured. Transaortic flow was obtained in apical 5-chamber view and LV ejection-time (LVET) was measured. LV myocardial performance-index (MPI) was calculated as (MVco–LVET)/LVET×100%.

Atrial Structural and Functional Remodeling: M-mode spectrum was recorded in parasternal long-axis view at the level of the aortic valve and left-atrial (LA) dimension at end-diastole (LADd) and systole (LADs) were measured. LA fractional-shortening (LA-FS) was calculated as (LADs–LADd)/LADs ×100%. Right-atrial (RA)-diameter (RAD) was measured in apical 4-chamber view. The average of 3 consecutive cardiac cycles on a simultaneously-recorded ECG was used for heart-rate measurement. Similar imaging-planes were used at follow-up studies.
**In Vivo Electrophysiology**

Rats were intubated and ventilated under 3%-isoflurane anesthesia. An octopolar catheter (1.9-F) with 0.5-mm interelectrode spacing (Scisense) was inserted into the RA via the right jugular vein for stimulation and recording. A custom-built computer-based stimulator was used to trigger a stimulus isolator (Grass Telefactor model SD9K) with selected stimulation-protocols. We recorded surface ECG, intracardiac electrograms and stimulus artifacts with IOX software (EMKA technologies, Paris, France) for monitoring and off-line analysis. Electrogram-recordings were filtered between 1 and 500 Hz. Atrial effective refractory periods (AERPs) were determined with twice-threshold 2-ms square-wave pulses, 15-stimulus drive-trains (S1) at 100-ms cycle-length (CL), followed by an S2 decremented in 2-ms intervals. AERP was the longest S1-S2 interval that failed to generate a response. AF was induced with up to 3 extrastimuli and if this failed to induce AF, with burst-pacing. Extrastimuli were applied with a 15-beat basic S1-S1 train at a 100-ms CL. S2 was set 10 ms above the AERP and S3s were applied from the AERP+60 ms down to refractoriness by 2-ms decrements. If S3s failed to induce AF, S4s were introduced with the same protocol as for S3s. If AF was not induced with 3 extrastimuli (S2S3S4), burst-pacing was applied at CLs starting at 100 ms and decreasing by 5-ms steps (100-pulse trains/CL) to the refractory period. Rapid and irregular atrial rate (>500 beats/min) with varying electrogram-morphology characterized AF. The maximum AF-duration in each animal was used to reflect the AF-substrate. The sinus node recovery time (SNRT) was the time from the last complex of a 30-second stimulus-train at a CL of 100 or 80 ms to the first spontaneous sinus beat. Corrected SNRT (cSNRT) was obtained by subtracting the spontaneous sinus-rhythm CL. The atrial CL at which 1:1 atrioventricular conduction was
lost defined the Wenckebach Cycle Length (WCL). At the end of experiments, hearts were excised and immersed into buffered formalin or snap-frozen in liquid-N<sub>2</sub> for further analysis.

**Histology**

Hearts were preserved in 10%-buffered formalin for paraffin-embedment. Serial atrial-tissue sections (12-μm thickness, 500-μm spacing) were stained with Masson’s Trichrome. Analysis of 400× magnification images (15-20 from 3-5 sections) was performed with Image-Pro 6.2 software. Perivascular areas were avoided. Fibrous-tissue content was expressed as a percentage of field-area and averaged across fields for each rat.

**Protein Extraction and Immunoblots**

Snap-frozen tissues were pulverized in liquid-N<sub>2</sub> and immersed in cold extraction buffer (25-mmol/L Tris-HCl, 1-mmol/L EGTA, 10%-glycerol, 1%-Triton-X100, 10-mmol/L dithiothreitol, 150-mmol/L NaCl, 5-mmol/L MgCl<sub>2</sub>, 25-mmol/L NaF, 1-mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1-μmol/L microcystin-LR, 10-μg/mL leupeptin, 10-μg/mL aprotinin, 1-μg/mL pepstatin and 0.1-mmol/L 4,2-aminoethyl-benzenesulfonyl-fluoride hydrochloride). The samples were homogenized at 12,000 rpm and incubated for 30 minutes on ice, then centrifuged for 10 minutes at 4°C, 11,000 rpm. Supernatants were collected and protein-concentrations estimated via Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Protein-extracts (100 μg) were denatured, separated by electrophoresis on 12% sodium-dodecylsulfate (SDS) polyacrylamide gels, and transferred to nitrocellulose membranes (0.45 A). Membranes were blocked with Tris-buffered saline (TBS)+5%-nonfat dry milk (NFDM) (90 minutes, room temperature) and incubated in 0.1%-Tween 80-TBS-NFDM primary
antibody overnight at 4°C. Membranes were then washed and incubated in 0.1%-Tween 80-TBS-NFDM peroxidise-conjugated secondary-antibody solution (60 minutes, room temperature). Membranes were washed and signal visualized by enhanced chemiluminescence with Quantity-One software (Bio-Rad). Data were normalized to GAPDH-band intensity from the same samples on the same membranes.

The following primary antibodies were used: mouse anti-phospho-P38 MAP-kinase, mouse anti-phospho-ERK1/2 MAP-kinase, mouse anti-P38 MAP-kinase, mouse anti-ERK1/2 MAP-kinase (1:2,000; Cell Signaling Technology, Danvers, Mass); mouse anti-GAPDH (1:10,000; RDI, Fitzgerald Industries). Secondary antibody was horseradish peroxidise-conjugated anti-mouse; Jackson ImmunoResearch laboratories, 1:10,000.

qPCR-Analysis

RNA was isolated with mirVana isolation-kits (Ambion/Life Technology, Carlsbad, CA). RNA-concentration was quantified via Nanodrop. Real-Time qPCR for miR-21 was performed with FAM-labelled fluorogenic Taqman-assay primers (Applied Biosystems, Foster City, CA) and Taqman Universal Master Mix (Applied Biosystems). qPCR was performed on an MX3000P unit (Stratagene, Agilent technologies, Santa Clara, CA). Relative quantifications were calculated with the comparative-threshold cycle method ($2^{-\Delta\Delta C_t}$), with snU6 as internal standard. qPCR for Sprouty-1, procollagen Ia (procol-I) and procollagen IIIa (procol-III) was performed with rat-specific Taqman primers (Applied Biosystems) and relative quantities ($2^{-\Delta C_t}$) calculated with the geometric mean of 3 reference genes (HPRT-1, GAPDH, β2-microglobulin) as internal standards.
**In Vivo miR-21 Knockdown**

Initial model-characterization pointed to a role of miR-21 in adverse remodeling. Experiments were therefore performed in 4 additional groups of rats: 1) sham-operated rats with miR-21 knock-down probe (KD21; Supplemental Figure 1) injected into LA-myocardium; 2) sham-operated rats injected with scrambled-control probe (Scr21); 3) MI-rats injected with KD21; 4) MI-rats injected with Scr21. The KD21-probe was a locked nucleic acid (LNA) 15-mer oligonucleotide (tcagtctgataagct) with a complete phosphorothioate backbone, HPLC-purified. The Scr21-probe (cgtctagccacctag), predicted bioinformatically not to interact with any other gene, was a negative control. Both constructs were custom-synthesized with locked-nucleotide chemistry by Exiqon (Vedbaek, Denmark). KD21 and Scr21 were dissolved in normal-saline solution at 5 μg/μL. Following thoracotomy, 10-μL solution was injected with a Hamilton micro-syringe (31-gauge-needle) into the LA-wall at 5 separate injection-points. In MI-rats, LAD-ligation was performed after completing LA-myocardial injections. In sham-rats, LA-myocardial injections were performed without LAD-ligation. Following 2 or 8-week post-MI periods, rats were submitted to echocardiography, *in vivo* electrophysiological study and cardiac excision for histology or qPCR.

**Statistical analysis**

Quantitative, normally-distributed variables are expressed as mean±SEM. Normal distribution of variables was assessed visually in a Q-Q plot and tested with Shapiro-Wilks test. Analyses of non-repeated measures data that were normally distributed and involved more than 2 groups were performed with analysis of variance models, in which group-homoscedasticity was confirmed with Levene’s test. Linear mixed effects modelling (MIXED procedure) was used for
repeated-measures data, and the covariance matrix was selected for each analysis amongst autoregressive-1, compound symmetry, Toeplitz and unstructured on the basis of the lowest Akaike’s Information Criterion (AIC) value. In both analysis of variance and mixed effects models the main factors and all their interactions were included. When a significant interaction was found, pairwise comparisons were performed with Student’s t-test and corrected with Bonferroni correction applied on the calculated p-value (p-values shown are t-test values multiplied by the number of possible pair-wise comparisons). When no significant interaction was found, main factor effects only were assessed. Two-group only comparisons were performed with Student’s t-test.

For AF duration and WMSI, criteria for normality were not met and a non-parametric Kruskal-Wallis test with posthoc Dunn’s test was used. Results are expressed as median and first and third quartile (Q1, Q3; values delimiting bottom 25% and top 25% of the distribution).

Statistical analyses were carried out with SPSS 19.0 (IBM, NY, USA) and GraphPad v5.0 (La Jolla, CA, USA). A $P<0.05$ was considered significant.

A detailed explanation of the statistical analysis in each figure follows:

**Figure 1A-E:** All echocardiographic parameters were analyzed separately. Analyses were carried out with a linear mixed effects model including time-point as a repeated main factor, surgery as non-repeated main factor and their interactions. On the basis of the lowest Akaike’s Information Criterion (AIC), autoregressive-1 covariance matrix was selected for panels A-D; compound symmetry was selected for panel E. Significant interaction at the $P<0.001$ level was found for LADd, LADs, LA-FS, LV-FAC, and at the $P<0.05$ level for E/Em. Post-hoc t-tests
were carried out with a Bonferroni correction (correction factor = 7). Significant $P$-values of pairwise comparisons (Sham vs MI) within each time-point and with the preceding time-point matched group (sham or MI) are shown.

**Figure 1G:** Analysis was carried out with an analysis of variance model including time-point and surgery as non-repeated main factors and their interaction. Significant interaction at the $P<0.001$ level was found. Post-hoc t-tests were carried out with a Bonferroni correction (correction factor = 4). Significant $P$-values of pairwise comparisons (Sham versus MI) are shown.

**Figure 2 (B-E):** Non-paired t-tests were performed separately for each protein within each time-point. All protein-samples for each time-point were processed on the same gel, and the gels for each time-point were separate (because of the limited number of samples that could be run on each gel). Valid inter-group comparisons could only be performed on samples run on the same gel, because inter-gel technical differences produced systematic band-intensity differences between different gels, thereby invalidating comparisons between samples run on different gels. For this reason, non-paired t-tests between samples of each group run on the same gel were the appropriate statistical comparison rather than an ANOVA on all samples from all gels.

**Figure 3 (A-C):** Non-paired t-tests were performed separately for each mRNA expression-level.

**Figure 4A:** Analysis was carried out with an analysis of variance model including post-surgery time-point, treatment (KD21 versus Scr21) and MI versus Sham as non-repeated main factors
and all their interactions. Significant (time-point × treatment) interactions at the $P<0.01$ level and (time-point × surgery) at the $P<0.05$ level were found. Post-hoc t-tests were carried out with a Bonferroni correction (correction factor = 4). Significant $P$-values of pairwise comparisons (Scr21 versus KD21; Sham vs MI) are shown for each time-point.

**Figure 4C:** Analyses were performed with Kruskal-Wallis with Dunn posthoc test (data were non-normally distributed). Significant $P$-values of pairwise comparisons (Scr21 versus KD21) are shown.

**Figure 5B:** Analysis was carried out with an analysis of variance including treatment (KD21 versus Scr21) and surgery (MI versus Sham) as main non-repeated factors and their interaction. Significant interaction (treatment × surgery) at the $P<0.001$ level was found. Post-hoc t-tests were carried out with a Bonferroni correction (correction factor = 4). Significant $P$-values of pairwise comparisons (Scr21 versus KD21 treatment within each surgery group [Sham or MI], and sham versus MI within each treatment) are shown.

**Figure 5C:** Analysis was carried out with an analysis of variance including time-point, surgery and probe as main non-repeated effects and all their interactions. No interactions were statistically significant but significant treatment (Scr21 versus KD21) and surgery (Sham versus MI) main effects were found. Scr21 versus KD21 differences are shown.

**Figure 6 (A-D):** Analysis was carried out with an analysis of variance including surgery (Sham versus MI), treatment (KD21 versus Scr21) and time-point post-surgery as non-repeated main
factors and all their interactions. Panels (A) and (B) showed significant (surgery × time-point) interaction, and pairwise comparisons (Sham versus MI) are shown within each time-point. Post-hoc t-tests were carried out with a Bonferroni correction (correction factor = 4). No significant differences were present for panels (C) and (D).

Figure 7 (B-D): Analyses were carried out with an analysis of variance model including surgery, treatment, and time-point as non-repeated main effects and all their interactions. For all variables (B, C and D), a significant surgery main factor (Sham versus MI) effect without significant interactions was found. Sham versus MI differences are shown.

Supplementary Figure 2 (A-D): Each echocardiographic parameter was analyzed separately. Analyses were carried out with a linear mixed effects model including time-point as a repeated factor and surgery as non-repeated main factor, and their interactions. Autoregressive-1 covariance matrix was used for all panels. Significant interaction at the $P<0.001$ level was found for RAD, LVDd and LVDs, and at the $P<0.05$ level for MPI. Post-hoc tests were carried out with a Bonferroni correction (correction factor = 7). Significant $P$-values of pairwise comparisons (Sham vs MI) within each time-point and with the preceding time-point matched group (sham, MI) are shown.

Supplementary Figure 2E: Significant Kruskal-Wallis with Dunn posthoc test (data were non-normally distributed). Significant $P$-values of pairwise comparisons (Sham versus MI) within each time-point are shown.
Supplementary Figure 3B: Significant Kruskal-Wallis with Dunn posthoc test (data were non-normally distributed). Significant P-values of pairwise comparisons (Sham versus MI) within each post-surgery time-point are shown.

Supplementary Figure 4: Analysis for each echocardiographic variable was carried out with an analysis of variance model including time-point post-surgery, surgery (MI versus Sham) and treatment as main non-repeated effects and all their interactions. Significant surgery (Sham versus MI) effect was found for all parameters and are shown. For WMSI, normality could not be demonstrated, and a significant Kruskal-Wallis test with Dunn posthoc test was found. Significant P-values of pairwise comparisons (Sham versus MI) within each time-point/treatment group are shown.

Table 1: Analysis for each hemodynamic measurement was carried out with an analysis of variance including time-point post-surgery (1 week, 2 weeks, 4 weeks and 8 weeks) and group (MI versus Sham) as main non-repeated effects and their interaction. No significant interaction was found for any of the parameters. Significant Sham versus MI effects were found for all parameters (peak systolic LV pressure at the 0.05 level, all the remaining measurements at the 0.001 level) and are shown.

Table 2: Analysis for each EP measurement was carried out with an analysis of variance including time-point post-surgery (2 weeks, 4 weeks and 8 weeks) and group (MI versus Sham) as main non-repeated effects, and their interaction. Significant group main effects (Sham versus MI) but no interactions between time and group were found for AERP_{100} (at the 0.001 level) and
WCL (at the 0.001 level) and differences are shown. Maximum AF duration was non-normally distributed and a Kruskal-Wallis test was carried out with Dunn’s post-hoc test.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: (A) Conserved sequence of mature miR-21 in different species. The seed sites (5’ 2-8 nucleotides) are highlighted in yellow. (B) Sequence of LNA antisense to miR-21 and LNA scrambled sequence as negative control. Note that the scrambled control is a random sequence designed by Exiqon and is predicted not to target any of the known miRNAs. The complimentary between KD21 and miR-21 is highlighted in yellow with “|” and the lack of complimentary between Scr21 and miR-21 is shown. The boldface and underlined letters represent the LNA-modified oligonucleotides.

Supplemental Figure 2: Echocardiographic results for right atrial dimension (A), LV measurements (B-D), and WMSI (E) in the model-characterization experiments. Results for A-D are mean±SEM; for E are median (Q1, Q3). Number of animals used are shown on the bars. Statistical analysis with linear mixed modeling (A-D) and Kruskal-Wallis test, posthoc comparisons with Dunn’s test (E). *P<0.05, **P<0.01, ***P<0.001 Sham versus MI in time-point and probe matched animals. Bsl=baseline; 2w, 8w= 2, 8 weeks post-surgery.

Supplemental Figure 3: (A) Example of an AF recording in an 8 week post-MI rat induced by an S1-S2-S3 protocol. Surface ECG, atrial electrogram (A-EGM) and stimulus artefacts (Stim.) were recorded simultaneously. (B) AF duration (median (Q1, Q3)) of induced episodes during the electrophysiological study. Analysis with Kruskal-Wallis with Dunn’s posthoc. **P<0.01 Sham versus MI. Times indicated on the horizontal axis are post-surgery.

Supplemental Figure 4: Echocardiographic measurements (mean±SEM), except for WMSI (median (Q1, Q3)) in Scr21- or KD21- treated rats. Statistical analysis with linear mixed effects model, except for WMSI (Kruskal-Wallis with post-hoc Dunn’s test). Number of animals used are shown in LADd results (same Ns for all measurements). LADd: left atrium diastolic
diameter, LADs: left atrium systolic diameter; FS: left atrium fractional shortening; RAD: right atrium diameter; LVDd: left ventricle diameter at the end of diastole; LVDs: left ventricle diameter at the end of systole; LV-FAC: left ventricular fractional area change; E/Em: E mitral wave amplitude to lateral Em amplitude ratio; WMSI: Wall motion score index. **$P<0.01$, ***$P<0.001$ Sham vs MI.
A
miR-21 in different species

5’-UGCUUAUCAGACUGAGUUGA-3’  Rat
5’-UGCUUAUCAGACUGAGUUGA-3’  Mouse
5’-UGCUUAUCAGACUGAGUUGA-3’  Canine
5’-UGCUUAUCAGACUGAGUUGA-3’  Human

B
Alignment of KD21 with miR-21

3’-TCTGATAGCTCTCCT-5’  KD21 (LNA anti-miR-21)
5’-UGCUUAUCAGACUGAGUUGA-3’  Rat (miR-21)

3’-ATGCACCTGCTCCT-5’  Scr21 (LNA anti-miR negative control)
5’-UGCUUAUCAGACUGAGUUGA-3’  Rat (miR-21)

Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4