Identification of Key Small Non-Coding MicroRNAs Controlling Pacemaker Mechanisms in the Human Sinus Node

Maria Petkova, PhD*; Andrew J. Atkinson, PhD*; Joseph Yanni, PhD; Luke Stuart, MRes; Abimbola J. Aminu, MRes; Alexandra D. Ivanova, MSc; Ksenia B. Pustovit, PhD; Connor Geragthy, MBChB; Amy Feather, MRes; Ning Li, PhD; Yu Zhang, PhD; Delvac Oceandy, PhD; Filip Perde, PhD; Peter Molenaar, PhD; Alicia D’Souza, PhD; Vadim V. Fedorov†; Halina Dobrzynski†

BACKGROUND: The sinus node (SN) is the primary pacemaker of the heart. SN myocytes possess distinctive action potential morphology with spontaneous diastolic depolarization because of a unique expression of ion channels and Ca2+-handling proteins. MicroRNAs (miRs) inhibit gene expression. The role of miRs in controlling the expression of genes responsible for human SN pacemaking and conduction has not been explored. The aim of this study was to determine miR expression profile of the human SN as compared with that of non-pacemaker atrial muscle.

METHODS AND RESULTS: SN and atrial muscle biopsies were obtained from donor or post-mortem hearts (n=10), histology/immunolabeling were used to characterize the tissues, TaqMan Human MicroRNA Arrays were used to measure 754 miRs, Ingenuity Pathway Analysis was used to identify miRs controlling SN pacemaker gene expression. Eighteen miRs were significantly more and 48 significantly less abundant in the SN than atrial muscle. The most interesting miR was miR-486-3p predicted to inhibit expression of pacemaking channels: HCN1 (hyperpolarization-activated cyclic nucleotide-gated), HCN4, voltage-gated calcium channel (Ca2+)1.3, and Ca2+3.1. A luciferase reporter gene assay confirmed that miR-486-3p can control HCN4 expression via its 3′ untranslated region. In ex vivo SN preparations, transfection with miR-486-3p reduced the beating rate by ≈35±5% (P<0.05) and HCN4 expression (P<0.05).

CONCLUSIONS: The human SN possesses a unique pattern of expression of miRs predicted to target functionally important genes. miR-486-3p has an important role in SN pacemaker activity by targeting HCN4, making it a potential target for therapeutic treatment of SN disease such as sinus tachycardia.

Key Words: ion channels ■ microRNAs ■ pacemaker of the heart ■ sinus node disease

The sinus node (SN) is the primary pacemaker of the heart and is located at the junction of the superior vena cava with the right atrium (RA). It is an extensive crescent shaped structure and its 3-dimensional anatomy has recently been shown within the whole ex vivo human heart.1–4 The SN myocytes possess distinctive action potential morphology, with a phase (phase 4) of slow, spontaneous, diastolic depolarization ultimately responsible for pacemaking. A unique expression of ion channels and Ca2+-handling proteins in the SN (described in the human by Chandler et al5) is responsible for 2 main mechanisms that synergistically generate this pacemaker potential during phase 4—the membrane voltage and Ca2+ clocks.6

Correspondence to: Halina Dobrzynski, PhD, University of Manchester, CTF building, 46 Grafton Street, Manchester M13 9NT, United Kingdom. E-mail: halina.dobrzynski@manchester.ac.uk

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*Dr Petkova and Mr Atkinson are co-first authors.
†Dr Fedorov and Dr Dobrzynski are co-last authors.
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Recent extensive work on non-coding molecules has begun to recognise their robust gene regulatory functions. One family of non-coding molecules are the microRNA (miR) family. miRs are ≈18 to 24 nucleotide single stranded RNAs, which regulate mRNA translation into functional protein through post-transcriptional repression by complementary nucleotide binding to the 3 prime untranslated region (3′UTR) recruiting the target gene into RNA-induced silencing complexes to be rendered translationally incompetent. miRs negatively regulate gene expression at the post-transcriptional level by degradation of mRNA or translational repression, and have been implicated in cardiac development and pathophysiological processes such as cardiac hypertrophy, fibrosis, arrhythmias, and heart failure. There is some evidence that miRs are involved in the SN function: in the mouse, the key pacemaker gene, HCN4 (hyperpolarization-activated cyclic nucleotide-gated 4), is under the control of miRs following athletic training. The aim of this work was to determine if differences in miR expression between the SN and neighboring atrial muscle in the human can explain the differences in the expression of pacemaker genes in the 2 tissues and why the SN shows pacemaking and the atrial muscle does not.

**METHODS**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Human Tissue**

The human specimens used in this study are described in detail in Table S1. They were obtained, dissected and frozen by co-authors in Australia (PM) and Romania (FP) under their local ethical approval procedures. No informed consent was required. Specimens were stored under the Human Tissue Act 2004.

Upon arrival of donor hearts to the Prince Charles Hospital (in a cardiologic solution on ice) and cadavers (10–35 hours from death) to National Institute of Legal Medicine right atrial/venocaval blocks (an example is shown in Figure 1C) were removed and trimmed, immediately frozen in liquid nitrogen or −60°C liquid isopentane and stored in freezers (−60° to −80°C). Specimens were transported on dry ice to the University of Manchester where the frozen samples were stored at −80°C.

**Animal Tissue**

Male Wistar-Hanover rats (Charles River UK Ltd.; 230–250 g) were euthanized in accordance with the guidelines of the Animal (Scientific Procedures) Act 1986 and the local ethical committee of the University of Manchester.

**Histology**

To identify the location of the SN, histology was performed on 10- to 30-μm thick cryosections, which were cut perpendicular to the crista terminalis from each specimen listed in Table S1. The cryosections were stained with Masson trichrome to identify the location of the SN as previously described (Figure 1).
Sections were fixed overnight at room temperature in Bouin solution (Sigma) and then rinsed 3 times in 70% alcohol for 10 minutes. Sections were: stained with Celestine blue for 5 minutes and washed in tap water for 10 minutes; stained with Cole alum hematoxylin for 10 minutes and washed for 15 minutes in tap water; stained with acid fuchsin for 5 minutes and washed for 30 minutes in tap water; and placed in phosphomolybdic acid for 5 minutes, drained and then stained with methyl green for 1 minute and washed for 20 minutes in tap water. Sections were then dehydrated using a graded series of alcohol as follows: 70% alcohol (1 minute), 90% alcohol (1 minute), 100% alcohol (twice for 2 minutes). Finally, sections were placed in histoclear solution for 5 minutes and mounted with distyrene, plasticizer, and xylene (Thermo Fisher). Histological sections were visualized with a light microscope (Zeiss LSM5) and an Axiocam camera (Zeiss) and collected with Axiovision software (Zeiss).

**Immunohistochemistry**

To further confirm the location of the SN, immunohistochemistry experiments were performed on sections neighbouring those used for histological verification. Tissue sections were fixed in 10% neutral buffer formalin (Sigma) for 30 minutes and washed...
3 times for 10 minutes in 0.01 mol/L PBS (Sigma). Tissue sections were then treated with 0.1% Triton X-100 for 30 minutes, washed 3 times for 10 minutes in PBS and then blocked with 1% bovine serum albumin (Sigma) in PBS for 1 hour at room temperature. Sections were then incubated overnight at room temperature with primary antibodies diluted in 1% bovine serum albumin in PBS. The antibodies used in immunohistochemical experiments are listed in Table S2. Sections were washed 3 times in PBS after incubation with the primary antibody and then incubated with a secondary antibody conjugated to fluorescence markers diluted 1:100 in 1% bovine serum albumin in PBS for 2 hours at room temperature. The sections were washed 3 times in PBS and mounted using Vectashield mounting medium (Vector Laboratories) and coverslips sealed. Immunofluorescence was detected by a confocal microscope (Zeiss LSM5, Zeiss Microscopy) and images were taken with Pascal software (Zeiss Microscopy).

Tissue Sampling and RNA Extraction

The SN area was identified by the presence of the SN artery, a large amount of connective tissue, positive staining for HCN4. After identification of the location of the SN by histology and immunohistochemistry, total RNA was isolated from small SN biopsies taken around the SN artery and an area of right atrial pectinate muscle remote from the SN and flash frozen in liquid N2.2 The tissue biopsies from the SN and remote from the SN and flash frozen were washed 3 times in PBS and then blocked with 1% bovine serum albumin (Sigma) in PBS for 1 hour at room temperature. Sections were then incubated overnight at room temperature with primary antibodies diluted in 1% bovine serum albumin in PBS. The antibodies used in immunohistochemical experiments are listed in Table S2. Sections were washed 3 times in PBS after incubation with the primary antibody and then incubated with a secondary antibody conjugated to fluorescence markers diluted 1:100 in 1% bovine serum albumin in PBS for 2 hours at room temperature. The sections were washed 3 times in PBS and mounted using Vectashield mounting medium (Vector Laboratories) and coverslips sealed. Immunofluorescence was detected by a confocal microscope (Zeiss LSM5, Zeiss Microscopy) and images were taken with Pascal software (Zeiss Microscopy).

Reverse Transcription, Preamplification, and Quantitative Polymerase Chain Reaction for miRs

One-hundred and eighty-five nanograms RNA from each sample were reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (ThermoFisher). The product of this reaction (2.5 μL) was preamplified with Megaplex PreAmp Primers (ThermoFisher). The primers were divided into pool A and B, each pool containing 380 stem-looped reverse transcription primers and TaqMan PreAmp Master Mix (Applied Biosystems) in a 25-μL polymerase chain reaction. The preamplification cycles were run as follows: 95°C (10 minutes), 55°C (2 minutes), and 75°C (2 minutes) followed by 12 cycles of 95°C (15 seconds) and 60° (4 minutes). The cDNA products for each sample were diluted to 100 μL with 0.1x Tris buffer and EDTA, ethylenediaminetetraacetic acid, molecule (pH=8.0). Ten nanoliters of the diluted cDNA were used for quantitative polymerase chain reaction (qPCR) using TaqMan Array Human MicroRNA A+B Cards Set v3.0 (ThermoFisher) for 754 human microRNAs. 7900HT Fast Real-Time PCR System (Applied Biosystems) was used for qPCR. The reaction conditions were as follows: 92°C for 10 minutes, 40 cycles of 97°C for 1 second, 60°C for 20 seconds. RQ Manager (Applied Biosystems) was used to obtain the average threshold cycle values. RealTime StatMiner (Integromics) was used for differential expression analysis. GeNorm stability assessment of the suitability of the housekeepers for the analysis of cards A and B was used. Housekeepers RNU44 and RNU48 were selected to analyze card A; RNU44 and U6 small nuclear RNA were used for card B. Statistical analysis of the expression levels was performed using Benjamini-Hochberg test and P<0.05 values were assumed as significant.

Ingenuity Pathway Analysis Bioinformatics

Sixty-six miRs listed in Table S3 were joined with the mRNA data for the SN and atrial muscle from the study by Chandler et al.5 and analyzed using Ingenuity Pathway Analysis (IPA, Qiagen) to identify potential interactions and relationships between the miRs and mRNAs involved in the membrane and Ca2+ clock pacemaker mechanisms. IPA uses data from TarBase database, miRecords (mirecords.biolead.org),
TargetScan (www.targetscan.org/) and ma22 (cm.jefferson.edu/ma22) to predict if any of the miRs potentially target the mRNAs based on conserved 8mer (≥0.8 conserved branch length) and 7mer sites that match the seed region of each miR.

Next Generation Sequencing
Next generation sequencing was performed at the Genomic Technologies Core Facility at the University of Manchester on RNA samples collected from frozen human SN preparations. SN samples were collected from the area around the SN artery and right atrial muscle samples from the pectinate muscles remote from the SN region. Samples were collected from 3 human specimens (Specimens 8, 9, 10 in Table S1). Quantity and integrity of the RNA samples were measured using a 2200 TapeStation (Agilent Technologies) to ensure their suitability. Subsequently, TruSeq Stranded mRNA assays (Illumina) were used to produce libraries of more stable, single-stranded cDNA as follows. Total RNA was purified to polyadenylated mRNA via magnetic separation technology, which works through hybridization of covalent interactions of oligo d(T)25 to poly (A) regions present in most eukaryotic mRNA. The mRNA sequences were fragmented into parts via divalent cations at higher temperature, and random primers were used to reverse transcribe the mRNA fragments into single-stranded cDNA. DNA polymerase and ribonuclease H-mediated the synthesis of the second cDNA strand produced from RNA oligonucleotides, originating from the 5’ end of the mRNA. The final cDNA library was generated by an addition of a single “A” base, binding of adapters to the fragments and purification and enrichment via a polymerase chain reaction. The cDNA libraries were incorporated into a multiplex system using the adapters; they were then pooled and clustered using a cBlot instrument (Illumina). Optical flow-cells containing the mRNA samples were then paired-end sequenced and mRNA was quantified through repeating 76 cycles twice, using a HiSeq4000 instrument (Illumina). Bcl2fastq software (2.17.1.14) was used to generate an mRNA expression database for each individual SN versus atrial muscle and calculate fold difference in expression between the 2 regions.

Luciferase Reporter Gene Assay
Rat cardiac H9C2 cells (ATCC, UK) were maintained in DMEM (Invitrogen), containing 10% fetal bovine serum, and 1% penicillin-streptomycin. H9C2 cells were seeded at a density of 5x10^5 cells per well in 24-well plates 24 hour before the transfection experiment. Cells were transfected with 500 ng HCN4 or CaV1.3 3’UTR-containing plasmid (see below for description of the plasmid) and 1 μg miR-486-3p, scrambled miR, or culture media only. Lipofectamine 2000 (Invitrogen, UK) transfection agent was used in accordance with the manufacturer’s instructions. The DNA-lipofectamine transfection complex was incubated for 20 minutes at room temperature and then 37°C and 5% CO2 for 24 hours, followed by washing with PBS and lysis via passive lysis buffer (Promega) on a rocker for 20 minutes. Luciferase reporter gene activity, which is directly proportional to mRNA expression of the target gene, was assessed using the luciferase assay system (Promega). A Lumat LB9507 luminometer (Berthold Technologies) was used to measure the bioluminescent activity in 10 μL cell lysate. Each assay was performed in quadruplicate and repeated 3 independent times. The luciferase assay activity was normalised to Renilla (Promega) activity and expressed as a ratio.

Sinus Node Preparations Used for Extracellular Potential Recording
Three-month-old male Wistar–Hannover rats weighing 230 to 250 g were used. Animals had free access to food and water and were maintained under standard laboratory conditions in a temperature-controlled room (22°C) with a 12:12 hour light:dark lighting regime. Animals were humanely euthanized by a Schedule 1 procedure (concussion and cervical dislocation) in accordance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986 under an institutional licence held at the University of Manchester.

Rats were humanely euthanised via CO2 inhalation and cervical dislocation. The heart was dissected out and placed in 37°C Tyrode’s solution (containing NaCl 120 mmol/L, CaCl2 1.2 mmol/L, KCl 4 mmol/L, MgSO4·7H2O 1.3 mmol/L, NaHCO3 25.2 mmol/L, NaH2PO4·2H2O 1.2 mmol/L, NaHCO3 25.2 mmol/L, glucose 5.8 mmol/L). The entire right atrium was then dissected and opened along the anterior atrial wall and anterior superior vena cava so that the posterior intercaval region remained intact (Figure 2A).

The dissection medium was changed to Modified Eagle’s Minimum Essential Medium, containing 5% fetal bovine serum (Life Technologies), and preparations incubated at 37°C/5% CO2. The culture medium was changed 8 hours after injection (see below) to Advanced DMEM/F-12 medium (Life Technologies), containing 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma). Preparations were kept for 24 hours.

Sinus Node Preparations Used for Sharp Microelectrode Recordings
Ten-week-old male Wistar rats weighing 280 to 300 g were used. Animals were kept in an animal house and
had free access to food and water and were main-
tained under standard laboratory conditions with a
12:12 hour light:dark lighting regime. Animals were hu-
manely culled. They were heparinized (100 IU/100 g,
intraperitoneal injection), anesthetized with isoflurane
(3.5%) and decapitated in accordance with European
Convention for the Protection of Vertebrate Animals
used for Experimental and other Scientific Purposes
(Council of Europe No 123, Strasbourg 1985) and
approved by the Ethics Committee of the National
Medical Research Center of Cardiology Institute of
Experimental Cardiology.

The chest was opened RA with intercaval SN region
was rapidly excised and pinned with endocardial side
up to the bottom of a 5 mL perfusion chamber filled with
physiological (Tyrode) solution of the following compo-
sition (in mmol/L): NaCl 118.0, KCl 2.7, NaH₂PO₄ 2.2,
MgCl₂ 1.2, CaCl₂ 1.2, NaHCO₃ 25.0, glucose 11.0, pH

Figure 2. Functional effects of microRNA-486-3p transfection of ex vivo rat
sinus node preparations.

A, Typical sinus node (SN) preparation. Site of injection around the SN artery shown. B, Beating rate of SN preparations in the 24 hours after injection with microRNA-486-3p or scrambled microRNA (means±SEM, n=9). C, Luciferase bioluminescence recorded 24 hours after transfection of H9C2 cells with 500 ng with HCN4 3 prime untranslated region -containing plasmid and 1 μg microRNA-486-3p or scrambled microRNA. As 2 control groups, cells were transfected with microRNA-486-3p alone or remained untransfected (ie, an equivalent volume of culture medium was added in place of transfected oligo or 3 prime untranslated region plasmid). These 2 control groups did not include the luciferase reporter. Means±SEM shown (n=3 batches of cells with 4 replicates; *P≤0.05). D and E, Quantitated polymerase chain reaction experiments showing expression of microRNA-486-3p, (D) and HCN4 (hyperpolarization-activated cyclic nucleotide-gated). (E) mRNA in SN preparations 24 hours after microRNA-486-3p transfection (means±SEM; n=4; *P≤0.05). CT indicates crista terminalis; HCN4, hyperpolarization-activated cyclic nucleotide-gated; miR, microRNA; SN, sinus node; and SVC, superior vena cava.
7.4±0.2 bubbled by 95% O₂ and 5% CO₂ gas mixture. The constant perfusion with flow rate of 10 mL/min at 37°C was started immediately after the preparation.

Spontaneously evoked SN pacemaker derived action potentials (APs) were recorded with glass microelectrodes (10–20 MΩ) filled with 3 mol/L KCl, connected to Warner intracellular electrometer (IE-210, Warner Instruments, USA) from the endocardial side of the preparations. Signal was digitized at 10 kHz sampling rate with analog-digital converter (E-154, ADC L-card, Russia). The rate of spontaneous AP was calculated using PowerGraph (PowerGraph 3.3 Professional, version 3.3.8, DISoft) and MiniAnalysis software (Synaptosoft, USA, version 6.0.7). The preparations were equilibrated for 30 minutes before recording of control APs in 3 to 4 mm² region surrounding SN artery bifurcation. Only APs with a diastolic depolarization (35–55 mV/s) and a slow rate of the AP upstroke (<15 V/s) were considered as pacemaker.

After the control recording, the SN region was injected with Tyrode solution (control), lipofectamine or miR-486-3p transfection mixture. The 5 µL of transfection mixture was delivered via glass microelectrode (tip diameter <50 µm) connected to Narishige micromanipulator and microinjection syringe pump (Harvard Apparatus, PHD ultra) with a constant rate 1 µL/min, the injection was repeated for 5 times to cover the 2 to 4 mm² of SN region and to deliver 25 µL of the transfection mixture in total. Pacemaker APs from the same sites in SN preparations were recorded for at least for 5 minutes immediately, 2, 4, and 6 hours after the injections. The rate of spontaneous APs as beats per minute was calculated.

Transfection Injection

=2 µL transfection mixture, containing 6 µL 1x Modified Eagle’s Minimum Essential Medium, reduced serum medium (Life Technologies), 1.5 µL Lipofectamine RNAmix (Life Technologies), and 2.5 µL miR-486-3p (MC12986; Life Technologies), was injected at the bifurcation of the SN artery with a 10 µL NanoFil syringe (World Precision Instruments). Control preparations were transfected with Cy3-labeled pre-miR negative control (ThermoFisher). All transfection was performed immediately after dissection of the preparations at the time of the initial culture. The beating rate was measured 24 hours later, ie, post injection.

Plasmids

Human HCN4 (NCBI Reference Sequence: NM_005477.2; HmiT088528-MT06) and Ca₃.1.3 (NCBI Reference Sequence: NM_000720.2; HmiT054373-MT06) 3’UTR-containing plasmids were purchased from GeneCopoeia (Rockville, MD, USA). The pEZX-MT06 luciferase miR expression vector contained reporter genes for luciferase and Renilla luciferase. The amplification of the plasmids was performed as follows: DH5α Escherichia coli cells (Sigma) were transformed with the plasmids. A single bacterial colony transfected with the plasmid was incubated in 2 mL LB medium, containing 100 µg/mL ampicillin overnight at 37°C, shaking at 150 rpm. Plasmid DNA was purified from the transduced E coli using PureLink Plasmid Kit (Thermo Fisher) according to the manufacturer’s protocol. Restriction digest, to confirm the presence of the correct ligation of the miR 3’UTR inserts in the pEZX-MT06 vector, was then performed. One microgram of each plasmid was incubated with restriction endonuclease enzymes EcoRV and HindIII (New England Biolabs) overnight at 37°C. DNA gel electrophoresis was then performed to confirm the presence of the expected DNA fragments.

Extracellular Potential Recording

To record and monitor automaticity of the ex vivo SN preparations, extracellular potentials were recorded from the right atrial appendage using 2 0.15-mm diameter stainless steel electrodes (ADInstruments) as previously described by Morris et al.17 In addition, the control medium surrounding the preparation was grounded with a 0.15 mm wire earth electrode. Extracellular potentials were continuously recorded using PowerLab 4/35, 4-channel recorder, and LabChart v7 software (ADInstruments). The average beating rate of the SN preparation was calculated via the detection of a deflection >2 SD of a paced beat. Recordings were collected for 24 hours and the SN preparation retained for further experiments.

Statistical Analysis

Mean±SEM values are shown. Significant differences were identified with 1-way ANOVA and/or paired t-tests. A difference was assumed to be significant at P≤0.05 or P≤0.001.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the article as written.

The presented data can be available from the corresponding author upon request.

RESULTS

Differential MicroRNA Expression in Human Sinus Node and Atrial Muscle

The expression profile of miRs in the SN and neighboring right atrial muscle was mapped using qPCR and a TaqMan assay for human miRs. Out of the 754 human miRs examined, 18 were significantly more
abundant (Figure 1A) and 48 significantly less abundant (Figure 1B) in the SN than the atrial muscle. The location of the SN tissue in which the miR expression was measured is shown in Figure 1C and 1D and the miRs that are significantly differentially expressed in the SN and atrial muscle are summarized in Table S3.

**Ingenuity Pathway Analysis**

IPA software was used to predict which of the miRs that are differentially expressed between the SN and atrial muscle may be involved in the differential expression of key ion channels, Ca2+-handling molecules and connexins involved in the regulation of the membrane, and Ca2+ clock pacemaker mechanisms. The predictions were based on miR-to-mRNA sequence binding probability, in combination with miR expression data for the human SN and atrial muscle from this study (Table S3) and expression of mRNA for key ion channels, Ca2+-handling proteins and connexins for the human SN, and atrial muscle from Chandler et al, 2009; upregulation of an miR is expected to lead to downregulation of its target mRNA and vice versa. TargetScan Human and/or TarBase software was used to predict target mRNA; rna22 software was used to predict the number of binding sites for an miR on an mRNA. The predicted miR-mRNA relationships are summarized in Table and are shown graphically.

### Table. Summary of Predicted MicroRNA-mRNA Interactions in the Human Sinus Node

| miR     | Expression of miR in Sinus Node (vs Right Atrial Muscle) | Predicted Target | Expression of Target mRNA in Sinus Node (vs Right Atrial Muscle) | No. of Binding Sites for miR on Predicted Target mRNA |
|---------|--------------------------------------------------------|------------------|-----------------------------------------------------------------|------------------------------------------------------|
| miR 1-3p| ↓                                                      | Tbx3             | ↑                                                               | 1                                                   |
|         |                                                       |                  | HCN1                                                            | 1                                                   |
|         |                                                       |                  | HCN4                                                            | 1                                                   |
| miR 10b-5p| ↑                                                | Gja5             | ↓                                                               | 1                                                   |
|         |                                                       | (Connexin 40)    |                                                                  |                                                     |
| miR 30c-5p| ↓                                                  | HCN1             | ↑                                                               | 1                                                   |
|         |                                                       |                  | HCN4                                                            | 1                                                   |
|         |                                                       |                  | Cacna1g, Ca,1.3                                                  | 1                                                   |
| miR 133a-3p| ↓                                              | HCN4             | ↑                                                               | 5                                                   |
| miR 153-3p| ↑                                                | Scn5a (Na,1.5)   | ↓                                                               | 1                                                   |
|         |                                                       |                  | Cacna1c (Ca,1.2)                                                | 1                                                   |
|         |                                                       |                  | RyR2                                                            | 2                                                   |
| miR 198| ↑                                              | Cacna1c (Ca,1.2) | ↓                                                               | 2                                                   |
|         |                                                       |                  | Kcnh2 (ERG)                                                     | 7                                                   |
|         |                                                       |                  | RyR2                                                            | 1                                                   |
| miR 204-5p| ↑                                               | Cacna1c (Ca,1.2) | ↓                                                               | 1                                                   |
| miR 215-5p| ↑                                               | Kcnha4 (K,1.4)   | ↓                                                               | 1                                                   |
| miR 371-3p| ↓                                               | Cacna1d (Ca,1.3) | ↑                                                               | 1                                                   |
|         |                                                       |                  | Gjc1 (Connexin 45)                                              | 5                                                   |
| miR 422a| ↓                                               | Tbx3             | ↑                                                               | 1                                                   |
| miR 429| ↓                                               | Tbx18            | ↑                                                               | 1                                                   |
| miR 486-3p| ↓                                           | HCN1             | ↑                                                               | 6                                                   |
|         |                                                       |                  | HCN4                                                            | 7                                                   |
|         |                                                       |                  | Cacna1d (Ca,1.3)                                                | 6                                                   |
|         |                                                       |                  | Cacna1g (Ca,3.1)                                                | 1                                                   |
| miR 512-5p| ↑                                           | Gja1 (Connexin 43) | ↓                                                               | 1                                                   |
| miR 938| ↓                                           | Cacna1d (Ca,1.3) | ↑                                                               | 9                                                   |
| miR 1225-3p| ↑                                           | Scn5a (Na,1.5)   | ↓                                                               | 2                                                   |

The data are based on the expression of miRs (microRNAs) from this study and the expression of selected mRNAs important for pacemaking from Chandler et al.5 The columns from left to right show the miRs, expression of the miRs in the sinus node vs the right atrial muscle, the gene names (ion channel names shown in parenthesis) of targets predicted by TargetScan Human and/or TarBase software, expression of the predicted targets in the sinus node vs the atrial muscle, and the number of potential binding sites on the target for each miR (predicted by rna22 software). Note that upregulation of an miR is expected to result in a downregulation of the target and vice versa. ERG indicates ether-a-go-go-related gene; Gja, gap junction protein alpha 5; HCN1, hyperpolarization-activated cyclic nucleotide-gated; miR, microRNA; RyR, ryanodine receptor 2; and Tbx, t-box.
in Figure 1 (arrows). Of the 18 miRs that were more abundant in the SN than the atrial muscle, 7 were predicted to target physiologically relevant pacemaker mRNAs that were expressed at lower levels in the SN compared with the atrial muscle (Figure 1 arrows; Table). The 7 miRs were: miR-10b-5p, predicted to target connexin 40; miR-153, predicted to target Cav1.2, ryanodine receptor 2, and Na\textsubscript{v}1.5; miR-198, predicted to target Ca\textsubscript{v}1.2, ryanodine receptor 2, and ether-a-go-go-related gene (ERG); miR-204, predicted to target Cav1.2; miR-215, predicted to target K\textsubscript{v}1.4; miR-512-5p, predicted to target connexin 43; and miR-1225-3p, predicted to target Na\textsubscript{v}1.5 (Figure 1, arrows). Of the 48 miRs that were more abundant in the atrial muscle than the SN, 8 were predicted to target physiologically relevant pacemaker mRNAs that were expressed at lower levels in the atrial muscle compared with the SN (Figure 1, arrows; Table). The 8 miRs were: miR-1-3p, predicted to target HCN1, HCN4 and T-box (Tbx)3; miR-30c, predicted to target HCN1 and Cav1.3; miR-133a, predicted to target HCN4; miR-371-3p, predicted to target Ca\textsubscript{v}1.3 and connexin 45; miR-422a, predicted to target Tbx3; miR-429, predicted to target Tbx18; miR-486-3p, predicted to target connexin 43; and miR-1225-3p, predicted to target Na\textsubscript{v}1.5 (Figure 1, arrows). Of the 48 miRs that were more abundant in the atrial muscle than the SN, 8 were predicted to target physiologically relevant pacemaker mRNAs that were expressed at lower levels in the atrial muscle compared with the SN (Figure 1, arrows; Table). The 8 miRs were: miR-1-3p, predicted to target HCN1, HCN4 and T-box (Tbx)3; miR-30c, predicted to target HCN1 and Cav1.3; miR-133a, predicted to target HCN4; miR-371-3p, predicted to target Ca\textsubscript{v}1.3 and connexin 45; miR-422a, predicted to target Tbx3; miR-429, predicted to target Tbx18; miR-486-3p, predicted to target connexin 43; and miR-1225-3p, predicted to target Na\textsubscript{v}1.5 (Figure 1, arrows). Of the 48 miRs that were more abundant in the atrial muscle than the SN, 8 were predicted to target physiologically relevant pacemaker mRNAs that were expressed at lower levels in the atrial muscle compared with the SN (Figure 1, arrows; Table). The 8 miRs were: miR-1-3p, predicted to target HCN1, HCN4 and T-box (Tbx)3; miR-30c, predicted to target HCN1 and Cav1.3; miR-133a, predicted to target HCN4; miR-371-3p, predicted to target Ca\textsubscript{v}1.3 and connexin 45; miR-422a, predicted to target Tbx3; miR-429, predicted to target Tbx18; miR-486-3p,
predicted to target Ca$\text{v}1.3$, HCN1, and HCN4; and miR-938, predicted to target Ca$\text{v}1.3$ (Figure 1, arrows).

**miR-486-3p Effect on SN Beating Rate**

Four miRs were predicted to target the pacemaker channel, HCN4, and the L-type Ca$^{2+}$ channel, Ca$\text{v}1.3$, (Table) and this may reflect the importance of these pacemaker channels. Three miRs were predicted to target the alternative pacemaker channel, HCN1, and the alternative L-type Ca$^{2+}$ channel, Ca$\text{v}1.2$ (Table). Two miRs were predicted to target the Na$^{+}$ channel, Na$\text{v}1.5$, the ryanodine receptor, ryanodine receptor 2, and the SN transcription factor, Tbx3 (Table). Finally, 1 miR was predicted to target a T-type Ca$^{2+}$ channel, Ca$\text{v}3.1$, 2 K$^{+}$ channels (Kv1.4 and ERG), 3 connexins (40, 43, and 45) and the SN transcription factor, Tbx18 (Table). Arguably the most important pacemaker channel in the SN is HCN4 and consequently it was of special interest. Of the miRs potentially targeting HCN4, miR-486-3p was of special interest, because it was predicted by IPA to have 7 binding sites on HCN4 mRNA in the human (Table). To confirm that miR-486-3p can target HCN4, experiments were performed on the ex vivo rat SN preparation. McGahon et al showed that the human miR-486-3p sequence is conserved in other species, including the rat. Also analysis showed that rat HCN4 mRNA has predicted binding sites for miR-486-3p. This suggests that the ex vivo rat SN preparation is suitable to validate HCN4 mRNA as a target of miR-486-3p. miR-486-3p (in a transfection mixture) was injected into the SN (Figure 2A) and the preparation was maintained in culture for 24 hours. Preparations injected with a non-functional scrambled miR were used as controls. miR-486-3p significantly reduced the beating rate of the ex vivo rat SN preparations by $\approx$35% 15 hours after transfection and this change was maintained over the rest of the 24-hour period of culture (Figure 2B). The effect of miR-486-3p on SN beating rate appeared to develop a few hours after transfection (Figure 2B).

In another set of experiments, using sharp micro-electrode recordings of the electrical activity in the...
Petkova et al. MicroRNAs Controlling Function of Human Pacemaker RA/SN preparations, we observed that after injections of the SN region (n=5) with miR-486-3p, the rate of the SN-derived spontaneous APs in the RA decreased gradually from 250±30 down to 32±21 beats per minute±SEM (Figure 3). This gradual reduction was observed from 2 hours after injection and was maximal at 6 hours post-injection. The rate of APs was only 13±9% (P<0.0001, n=5) of the initial rate before injections calculated both on the basis of the SN or RA recordings (Figure 3). After injections with Tyrode (n=5) and lipofectamine (n=5) there was no change to the rate of the SN-derived spontaneous APs (Figure 3).

**miR-486-3p Effect on HCN4 Expression**

To investigate whether miR-486-3p can affect HCN4 expression as predicted, a luciferase reporter gene assay was performed. The predicted binding sites for miR-486-3p are in the 3′-UTR of human HCN4. Therefore, the 3′-UTR of human HCN4 was introduced as the 3′-UTR of the luciferase gene. The resulting plasmid was transfected into rat cardiac H9C2 cells. Following transcription and translation, the expression of the luciferase protein (surrogate of HCN4 expression) was measured by the resulting luciferase bioluminescence. Bioluminescence was significantly less from cells transfected with the plasmid and miR-486-3p than from cells transfected with the plasmid and scrambled (non-functional) miR (Figure 2C). As expected, bioluminescence was also low if the cells were not transfected with the HCN4 plasmid but only transfected with miR-486-3p or neither transfected with the plasmid nor miR-486-3p (Figure 2C). This suggests that miR-486-3p can control HCN4 expression.

To confirm that miR-486-3p can affect HCN4 expression, experiments were conducted on the ex vivo rat SN preparation. Preparations were injected (Figure 2A) with miR-486-3p or scrambled miR. As expected, miR-486-3p expression was greater in preparations in which the SN was transfected with miR-486-3p rather than scrambled miR (Figure 2D). HCN4 mRNA expression was significantly reduced in preparations injected with miR-486-3p (as compared with preparations injected with the scrambled miR).
This shows that miR-486-3p controls HCN4 mRNA expression in the sinus node (miR-486-3p must promote HCN4 mRNA degradation). In the same ex vivo rat SN preparations, HCN4 protein expression was assessed by immunolabeling of HCN4 protein in thin cryosections through the SN (Figure 4A, 4B, and 4E). Labeling was significantly reduced in preparations injected with miR-486-3p (as compared with preparations injected with the scrambled miR) (Figure 4A, 4B, and 4E). Cell membrane preservation and integrity over the 24-hour incubation period was confirmed by Caveolin-3 immunolabeling (Figure 4C, 4D, and 4F).

Transcription Factors are Also Involved in Differential Gene Expression Between the Human Sinus Node and Atrial Muscle
Differential gene expression between the human sinus node and atrial muscle will not exclusively be the result of a differential expression of miRs—a differential expression of transcription factors will also be involved. Expression of transcription factors was investigated using next generation sequencing. The transcriptomes of 3 human SN and corresponding right atrial muscle samples were sequenced. The expression of the 3060 most abundant human mRNAs was investigated and principal component analysis confirmed distinct SN and atrial muscle mRNA profiles (Figure 5A); 1238 mRNAs had significantly higher expression in the SN (log2 fold change >1, \( P<0.05 \)), 1357 had significantly higher expression in the atrial muscle (log2 fold change <1, \( P<0.05 \)), and 465 were not significantly different between the 2 tissues (−1 <log2 fold change >1) (Figure 5B, red dots). IPA software was used to identify transcription factors and potential relationships with either miRs or mRNAs in this study; 68 transcription factors were significantly more expressed in the SN, and 60 were more significantly expressed in the atrial muscle. Six of the differentially expressed transcription factors have potential relationships with either the miRs...
or mRNAs in this study. These are Islet1, short stature homeobox 2, Tbx3, and Tbx18, which were significantly more highly expressed in the SN, and Tbx5 and NK2 homeobox 5, which were significantly more highly expressed in the atrial muscle (Figures 5B and 6). The predicted relationships between these transcription factors and either miRs or mRNAs are shown in Figure 7. This network shows 24 potential links (direct or indirect) between miRs and ion channels, but only 3 between transcription factors and ion channels. This raises the question of whether much of the ion channel regulation occurs at the post-transcriptional level via miRs. However, miRs themselves are under the control of transcription factors as shown in Figure 7.

**DISCUSSION**

This study shows, for the first time, a distinct expression pattern of miRs in the human SN compared with that of the right atrial muscle that is predicted to affect the expression of target molecules responsible for the pacemaker mechanisms in the heart. This study shows 66 differentially expressed miRs (Figure 1). The differentially expressed miRs are predicted to target mRNAs that have been reported to be differentially expressed in these tissues.\(^5\)

In this study, we found 7 miRs (miR-10b-5p, miR-153-3p, miR-198, miR-204-5p, miR-215-5p, miR-512-5p, and miR-1225-3p) expressed at higher levels in the SN compared with atrial muscle that are predicted to bind to and thus downregulate molecules that are known to be expressed at lower levels in the SN\(^5\): the Na\(^+\) channel, Na\(_v1.5\), the L-type Ca\(^{2+}\) channel, Ca\(_v1.2\), 2 K\(^+\) channels (Kv1.4 and ERG), the RYR2 (ryanodine receptor 2), and 2 connexins (40 and 43). The absence of Na\(_v1.5\) (and the corresponding absence of the Na\(^{+}\) current, \(I_{\text{Na}}\)) in the SN explains why the upstroke of the SN action potential is slow and the absence of the 2 high conductance connexins, 40 and 43, in the SN explains the poor electrical coupling in the SN (essential to protect the SN from the hyperpolarizing influence of the neighboring atrial muscle).\(^19\)

Eight miRs (miR-1-3p, miR-30c-5p, miR-133a-3p, miR-371-3p, miR-422a, miR-429, miR-486-3p, and miR-938) that were expressed at higher levels in the atrial muscle compared with the SN are predicted to inhibit molecules that are important for pacemaking: 2 pacemaker HCN channels (HCN1 and HCN4), 2 pacemaker Ca\(^{2+}\) channels (Ca\(_v1.3\) and Ca\(_v3.1\)), and the low conductance connexin isoform, connexin 45. If the predicted actions of the miRs are correct, this helps to explain why the atrial muscle does not normally show pacemaker activity; conversely, the low
expression of these miRs in the SN helps to explain why the SN does show pacemaking. Of these miRs and their predicted effects, miR-486-3p and its predicted effect on HCN4 were the focus, because of the importance of HCN4 and the number of predicted binding sites on HCN4 for miR-486-3p. A luciferase reporter gene assay confirmed that miR-486-3p can potentially control expression of HCN4 (Figure 2), and miR-486-3p, when ectopically expressed in the SN, reduced HCN4 mRNA and protein levels (Figures 2 and 4), and reduced the SN beating rate (Figure 2) In a recent study, we showed that miR-486-3p is upregulated in the SN in a mouse model of athletic training. Following athletic training, the downregulation of HCN4 and the corresponding ionic current (I\textsubscript{f}) in the SN and the consequent sinus bradycardia was attributed to an upregulation of miR-486-3p and miR-423-5p; in this study, a luciferase reporter gene assay showed that miR-486-3p was able to regulate mouse HCN4 expression—therefore, miR-486-3p is able to regulate both human (this study) and mouse HCN4 expression. In this study, we also identified key “embryonic” transcription factors that are differentially expressed in the adult human SN versus atrial muscle and with predicted links to either the miRs or ion channels of interest in the SN (Figures 5, 6, and 7). This suggests that both transcription factors and miRs are responsible for the unique gene expression pattern of the SN. Furthermore, transcription factors may frequently act via miRs.

CONCLUSIONS

The human SN possesses a unique pattern of expression of miRs. Some of the differentially expressed miRs are predicted to target genes that are important for pacemaking, such as HCN1, HCN4, Ca\textsubscript{i,1.3}, and Ca\textsubscript{3.1}. The action of miRs is complex with interactions between multiple miRs, transcription factors, and target genes. It has been confirmed that miR-486-3p has an important role in regulating SN pacemaker activity, miR-486-3p directly inhibits HCN4 and thereby reduces action potential generation by the SN, making it a potential target for manipulating pacemaking in therapeutic treatment of sinus node disease. For example, inappropriate sinus node tachycardia is currently treated using ivabradine to block HCN4\textsuperscript{20} and use of miR-486-3p could be alternative strategy. This study provides novel insights into the mechanisms controlling SN gene expression vital for its role as the primary pacemaker in the heart.

ARTICLE INFORMATION

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SUPPLEMENTAL MATERIAL
### Table S1. Human specimen information.

| Number | Age | Sex | Cause of death         | RNA amount (ng/µl) | RNA quality (RIN) | Use       |
|--------|-----|-----|------------------------|--------------------|-------------------|-----------|
| 1      | 29  | M   | Road accident          | 100.60/231.90      | 6.1/7.6           | qPCR      |
| 2      | 22  | M   | Road accident          | 30.98/301.40       | 8.0/7.7           | qPCR      |
| 3      | 66  | M   | Suicide                | 47.48/285.70       | 5.2/4.7           | qPCR      |
| 4      | 19  | M   | Suicide                | 114.11/389.00      | 6.4/7.2           | qPCR      |
| 5      | 60  | M   | Sudden death           | 25.4/123.25        | 7.2/6.5           | qPCR      |
| 6      | 54  | M   | Intracranial haemorrhage| 164.67/400.00  | 8.3/8.8           | qPCR      |
| 7      | 42  | M   | Subarachnoid haemorrhage| 173.65/423.00 | 8.5/9.1           | qPCR      |
| 8      | 19  | M   | Suicide                | 114.11/389.00      | -                 | NGS       |
| 9      | 21  | M   | Suicide                | 35.3/59.66         | -                 | NGS       |
| 10     | 54  | M   | Intracranial haemorrhage| 45.35/91.77 | -                 | NGS       |

- data not available but total RNA isolated from samples 8-10 passed their quality for NGS experiments
Table S2. Summary of primary antibodies.

| Protein   | Company   | Catalogue # | Source   | Dilution |
|-----------|-----------|-------------|----------|----------|
| Cx43      | Millipore | MAB3068     | Mono - Ms| 1:50     |
| HCN4      | Alomone   | APC-052     | Poly - Rbt| 1:50    |
| Caveolin3 | Transduction | 310421   | Poly-Ms     | 1:50     |
Table S3. Significantly up- or down- regulated miRs in the SN vs. RA. Mean relative miR expression and SEM in SN and RA, fold change and log fold change, and P values shown.

| miR         | SN mean | SN SEM | RA mean | RA SEM | Fold change | Log fold change | P value |
|-------------|---------|--------|---------|--------|-------------|-----------------|---------|
| hsa-miR-153-3p | 2.00E-05 | 1.00E-05 | 4.00E-06 | 3.00E-06 | 1.00E-05 | 5.00E-06 | 5.00E-02 |
| hsa-miR-104-5p | 8.10E-01 | 2.10E-01 | 1.60E-01 | 3.00E-02 | 5.50E-01 | 7.00E-01 | 2.00E-03 |
| hsa-miR-215-3p | 3.00E-03 | 1.00E-03 | 2.00E-04 | 1.00E-04 | 3.00E-03 | 1.23E+00 | 1.00E-03 |
| hsa-miR-577-3p | 6.00E-01 | 1.00E-05 | 1.47E+00 | 4.00E-06 | 7.80E-01 | 3.50E-01 | 4.00E-02 |
| hsa-miR-422a | 3.00E-03 | 1.00E-03 | 2.00E-03 | 1.00E-03 | 3.00E-03 | 4.00E-01 | 2.00E-02 |
| hsa-miR-429 | 2.00E-04 | 2.00E-04 | 2.00E-03 | 1.00E-03 | 2.00E-03 | 9.10E-01 | 4.00E-02 |
miRs with significant higher expression in SN highlighted in bold.

| miR   | SN mean  | SN SEM  | RA mean | RA SEM  | Fold change | Log fold change | P value |
|-------|----------|---------|---------|---------|-------------|-----------------|---------|
| hsa-miR-450b-3p | 1.40E-02 | 4.00E-03 | 3.00E-02 | 1.00E-02 | -3.00E-02 | -3.00E-01 | 4.00E-03 |
| hsa-miR-483-3p  | 6.00E-01 | 2.00E-03 | 1.00E-02 | 2.00E-03 | -7.00E-03 | -3.30E-01 | 5.00E-02 |
| hsa-miR-486     | 9.00E-04 | 2.00E-04 | 1.00E-03 | 2.00E-04 | -9.00E-04 | -5.70E-01 | 1.00E-02 |
| hsa-miR-480-3p  | 1.85E+01 | 1.16E+01 | 1.00E-05 | 6.00E-06 | 1.85E+01 | 6.10E+00 | 3.00E-02 |
| hsa-miR-513-3p  | 3.00E-07 | 1.00E-07 | 3.00E-06 | 2.00E-06 | -3.00E-06 | -9.70E-01 | 4.00E-02 |
| hsa-miR-523d-3p | 9.00E-05 | 4.00E-05 | 2.00E-04 | 3.00E-05 | -8.00E-05 | -2.70E-01 | 5.00E-02 |
| hsa-miR-520-3p  | 6.00E-04 | 1.00E-05 | 6.00E-06 | 2.00E-05 | -3.00E-05 | -8.40E-01 | 5.00E-02 |
| hsa-miR-514-3p  | 5.00E-06 | 2.00E-06 | 4.00E-04 | 2.00E-04 | -4.00E-04 | -1.95E+00 | 4.00E-02 |
| hsa-miR-548b-3p | 1.00E-04 | 1.00E-04 | 1.00E-04 | 6.00E-05 | 6.00E-05 | 2.80E-01 | 4.00E-02 |
| hsa-miR-548K    | 2.00E-09 | 1.00E-09 | 6.00E+00 | 8.00E-08 | 2.00E-09 | 7.20E-01 | 5.00E-02 |
| hsa-miR-171     | 8.00E-05 | 2.00E-05 | 4.00E-04 | 2.00E-04 | -3.00E-04 | -6.60E-01 | 3.00E-02 |
| hsa-miR-584-3p  | 6.00E-04 | 1.00E-04 | 1.00E-03 | 1.00E-04 | -9.00E-04 | -2.70E-01 | 4.00E-02 |
| hsa-miR-616-3p  | 2.00E-04 | 4.00E-05 | 3.00E-04 | 4.00E-05 | -1.00E-04 | -2.60E-01 | 4.00E-02 |
| hsa-miR-678-3p  | 3.00E-03 | 1.00E-03 | 3.00E-03 | 1.00E-03 | -2.00E-03 | -2.70E-01 | 4.00E-02 |
| hsa-miR-642a-5p | 3.00E-03 | 1.00E-03 | 3.00E-03 | 1.00E-02 | -2.00E-02 | -1.00E+00 | 4.00E-04 |
| hsa-miR-651-5p  | 2.00E-05 | 1.00E-05 | 4.00E-05 | 9.00E-06 | -2.00E-05 | -3.70E-01 | 4.00E-02 |
| hsa-miR-615-3p  | 1.00E-04 | 1.00E-04 | 1.00E-04 | 2.00E-07 | 6.00E-08 | 2.18E+00 | 3.00E-02 |
| hsa-miR-668-3p  | 5.00E-05 | 2.00E-05 | 4.00E-07 | 8.00E-08 | 5.00E-05 | 2.11E+00 | 3.00E-02 |
| hsa-miR-885-3p  | 1.00E-01 | 1.00E-03 | 4.00E-07 | 7.00E-08 | 1.00E-03 | 3.45E+00 | 2.00E-02 |
| hsa-miR-123-3p  | 2.00E-06 | 1.00E-06 | 1.00E-05 | 1.00E-06 | -1.00E-05 | -6.60E-01 | 3.00E-03 |
| hsa-miR-1225-3p | 3.00E-02 | 1.00E-02 | 1.00E-02 | 1.00E-02 | 1.00E-02 | 3.10E-01 | 3.00E-02 |
| hsa-miR-1233-3p | 5.00E-04 | 5.00E-04 | 5.38E-00 | 6.38E+00 | -5.38E+00 | -4.05E+00 | 3.00E-02 |
| hsa-miR-1244-3p | 5.00E-04 | 1.00E-04 | 1.00E-04 | 4.00E-03 | 2.09E-04 | 4.50E-01 | 1.00E-02 |