Fibroblast Inward-Rectifier Potassium Current Upregulation in Profibrillatory Atrial Remodeling

Xiao-Yan Qi¹, Hai Huang¹, Balazs Ordog¹, Xiaobin Luo¹, Patrice Naud¹, Yiguo Sun¹, Chia-Tung Wu¹,³, Kristin Dawson¹,³, Artavazd Tadevosyan¹, Yu Chen¹,², Masahide Harada¹,³, Dobromir Dobrev² and Stanley Nattel¹,³

X-Y.Q. and H.H. contributed equally to this study.

¹Research Center, Montreal Heart Institute, and Université de Montréal, Montreal, Quebec, Canada; ²Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada; ³Chang-Gung Memorial Hospital and University, Taoyuan, Taiwan, Republic of China; ⁴Department of Cardiology, Fujita Health University School of Medicine, Toyoake, Japan, and; ⁵Institute of Pharmacology, Faculty of Medicine, University Duisburg-Essen, Essen, Germany.

Running title: Atrial Fibroblast Iₜ,K and Remodeling in CHF

Subject codes:
[5] Arrhythmias, clinical electrophysiology, drugs
[132] Arrhythmias-basic studies
[152] Ion channels/membrane transport

Address correspondence to:
Stanley Nattel
5000 Belanger St. E.
Montreal
Quebec H1T 1C8
Canada
Tel.: 514-376-3330
Fax: 514-376-1355
stanley.nattel@icm-mhi.org

In December 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.47 days.
ABSTRACT

Rationale: Fibroblasts (FBs) are involved in cardiac arrhythmogenesis, and contribute to the atrial fibrillation (AF) substrate in congestive heart failure (CHF) by generating tissue-fibrosis. FBs display robust ion-currents, but their functional importance is poorly understood.

Objective: To characterize atrial FB inward-rectifier K⁺-current (I\textsubscript{K1}) remodeling in CHF and its effects on FB properties.

Methods and Results: Freshly-isolated left atrial (LA) FBs were obtained from control and CHF (ventricular tachypacing) dogs. Patch-clamp was used to record resting membrane potential (RMP) and I\textsubscript{K1}. RMP was significantly increased by CHF (from -43.2±0.8 mV, control, to -55.5±0.9 mV). CHF upregulated I\textsubscript{K1} (e.g at -90 mV from -1.1±0.2 pA/pF to -2.7±0.5 pA/pF), and increased expression of KCNJ2 mRNA (by 52%) and protein (by 80%). Ba\textsuperscript{2+} (300-µmol/L) decreased RMP and suppressed the RMP difference between CTL and CHF. Store operated Ca\textsuperscript{2+}-entry (Fura-2 AM) and FB proliferation (flow cytometry) were enhanced by CHF. Lentivirus-mediated overexpression of KCNJ2 (KCNJ2-OE) enhanced I\textsubscript{K1} and hyperpolarized FBs. Functional KCNJ2-suppression by lentiviral-mediated expression of a dominant negative KCNJ2-construct (KCNJ2-DN) suppressed I\textsubscript{K1} and depolarized RMP. KCNJ2-OE increased Ca\textsuperscript{2+}-entry and FB proliferation whereas KCNJ2-DN had opposite effects. Fibroblast-hyperpolarization to mimic CHF-effects on RMP enhanced Ca\textsuperscript{2+}-entry. MicroRNA-26a, which targets KCNJ2, was downregulated in CHF FBs. Knockdown of endogenous microRNA-26 to mimic CHF-effects upregulated I\textsubscript{K1}.

Conclusions: CHF upregulates fibroblast KCNJ2 expression and currents, thereby hyperpolarizing RMP, increasing Ca\textsuperscript{2+}-entry and enhancing atrial FB proliferation. These effects are likely mediated by microRNA-26a downregulation. Remodeling-induced fibroblast KCNJ2 expression-changes may play a role in AF promoting FB remodeling and structural/arrhythmic consequences.

Keywords: Fibroblast function, cardiac arrhythmias, heart fibrosis, calcium handling, arrhythmia mechanisms.

Nonstandard Abbreviations and Acronyms:
AMO Anti-miR oligonucleotide
α-SMA α smooth muscle actin
BCL Basic cycle length
BSA Bovine serum albumin
CHF Congestive heart failure
CTL Control
ECM Extracellular matrix
ERP Effective refractory period
FB Fibroblast
GFP Green fluorescent protein
KCNJ2-DN Dominant negative Kir2.1
KCNJ2-OE Overexpression Kir2.1
LA Left atrial
LV Left ventricular
RAA Right atrial appendage
RMP Resting membrane potential
INTRODUCTION

Congestive heat failure (CHF) is a very important cause of atrial fibrillation (AF), with structural remodeling, particularly tissue fibrosis, playing a central role. Fibroblasts are the most abundant cells in the heart. Fibroblast proliferation and differentiation into myofibroblasts is an important contributor to arrhythmogenesis under conditions like CHF, by enhancing the production of extracellular matrix (ECM) proteins like collagen and possibly via electrical interactions with cardiomyocytes.

Fibroblasts are known to express a wide range of ion-channels, but their functional role is poorly understood. Cardiac fibroblasts are not electrically excitable, but have polarized resting membrane potentials (RMPs), with average values as negative as -37 mV. The primary determinant of fibroblast RMP is the inward-rectifier K⁺-current, I_K1. We have recently shown that voltage-gated (Kv) K⁺-currents in cardiac fibroblasts are remodeled in CHF. The present study aimed to characterize changes in cardiac fibroblast I_K1 in CHF, and to define underlying mechanisms and potential functional significance.

METHODS

Animal model.
Adult mongrel dogs (22-30 kg) were divided into 2 groups: control (28 male, 2 female) and 2 week ventricular tachypacing-induced congestive heart failure (CHF; 21 male, 3 female). CHF dogs had unipolar pacing leads inserted fluoroscopically into the right ventricular apex, which were programmed at 240 bpm for 2 weeks. On study days, dogs were anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV, followed by 29.25 mg/kg per hour) and ventilated mechanically. Effective refractory periods (ERPs) were measured at basic cycle lengths (BCLs) of 150, 200, 250, 300 and 350 ms in the right-atrial appendage, with 10 basic stimuli (S1) followed by a premature extrastimulus (S2) with 5-ms decrements. AF was induced with atrial burst-pacing at 50 Hz and 10 V. Mean AF duration was based on 10 AF inductions in each dog. If the mean duration of the first five episodes of AF was longer than 2 minutes, AF was only induced five times. In 4 dogs/group, tissue sections were analyzed for fibrous-tissue content as previously described, by an investigator blinded to group assignment.

Fibroblast isolation and culture.
Atrial fibroblasts were obtained from left atria of adult mongrel dogs as described previously. Hearts were removed after intra-atrial injection of heparin (10,000 U), immersed in 2 mmol/L Ca²⁺ containing Tyrode’s solution, the left coronary artery was cannulated, and left atrial tissue perfused with 2 mmol/L-Ca²⁺ Tyrode’s solution (37°C, 100% O₂), then with Ca²⁺-free Tyrode’s solution (~10 minutes), followed by ~60-minute perfusion with the same solution containing collagenase (~0.48 mg/mL, CLSII, Worthington) and 0.1% bovine serum albumin (BSA, Sigma). Cells were dispersed by trituration in KB solution (when used for electrophysiological study or sample-acquisition for mRNA and/or protein analysis) or Medium 199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin and streptomycin for culture. Filtration (500 nm nanomesh) was used to remove debris, and cells were then centrifuged at 54.6×G for 5 minutes to pellet cardiomyocytes. The supernatant was collected and filtered through 50 μm nanomesh and centrifuged at 314.5×G for 10 minutes to concentrate fibroblasts. Freshly-isolated fibroblasts were then separated; one aliquot was flash-frozen in liquid-N₂ and stored for biochemical studies, and the remaining cells were cultured on non-coated glass coverslips. Fibroblasts were incubated in 5% CO₂/95% O₂ humidified air (37°C). A medium change was performed 4 hours after plating to remove any dead cells and debris, and medium was changed every 24 hours.
Ionic current and RMP recording.
All in vitro recordings were obtained at 37°C. The whole-cell perforated-patch technique was used to record RMP in current-clamp mode and tight-seal patch-clamp was used to record \(I_{K1}\) in voltage-clamp mode. Borosilicate glass electrodes filled with pipette solution were connected to a patch-clamp amplifier (Axopatch 200A, Axon). Electrodes had tip resistances of 6-8 MΩ. Nystatin-free intracellular solution was placed in the tip by capillary action (~30 s), then pipettes were back-filled with nystatin containing (600 μg/mL) pipette solution. \(I_{K1}\) was recorded as the 300-μmol/L \(Ba^{2+}\)-sensitive current. Tyrode’s solution contained (mmol/L) NaCl 136, CaCl₂ 1.8, KCl 5.4, MgCl₂ 1, NaH₂PO₄ 0.33, dextrose 10, and HEPES 5, titrated to pH 7.3 with NaOH. The pipette solution for RMP and \(I_{K1}\) recording contained (mmol/L) GTP 0.1, potassium-aspartate 110, KCl 20, MgCl₂ 1, MgATP 5, HEPES 10, sodium phosphocreatine 5, and EGTA 0.005 (pH 7.4, KOH). Junction potentials between bath and pipette solutions averaged 10.5 mV and were corrected for RMP measurements. Currents are expressed as densities (pA/pF), to control for changes in cell size/capacitance with CHF.

\(\text{Ca}^{2+}\) imaging.
One-day cultured canine atrial fibroblasts on microscope cover slips were loaded with Fura-2-acetoxymethyl ester (Fura-2 AM, 5 μmol/L, Invitrogen) in phenol-free M199 medium in the presence of Pluronic F-127 (20% solution in dimethylsulfoxide, 2.5 μg/mL) for 30 minutes at 36°C in a humidified incubator with 95% air/5% CO₂. Cover slips were fixed in a perfusion chamber on the stage of a microscope, and fibroblasts were superfused with 1.8 mmol/L \(Ca^{2+}\) Tyrode’s solution and maintained for at least 25-30 minutes at room temperature before experimental protocols to allow for deesterification of Fura-2 AM. Fura-2 was excited with dual excitation-wavelengths at 340 and 380 nm and emission was recorded at a wavelength of 510 nm. \(Ca^{2+}\)-imaging was obtained with an IonOptix Fluorescence System mounted on an upright Nikon FN-1 microscope. To measure store-operated \(Ca^{2+}\)-entry, cells were first exposed to \(Ca^{2+}\)-free solution for 10 minutes. \([Ca^{2+}]_o\) was then increased to 10 mmol/L to measure \(Ca^{2+}\)-entry via store-operated channels activated by \(Ca^{2+}\)-store depletion.

Cell proliferation and cell cycle analysis.
Cell cycle was analyzed with flow cytometry as previously described. Atrial fibroblasts were seeded at 4,000 cells/cm² in T-25 culture flasks (1.0 x10⁵ cells/flask, 25 cm² growth area) and were cultured for 3 days in M199 medium supplemented with 10% fetal bovine serum. The culture medium was replaced with lentivirus-containing medium for each group on day 2. Cells were harvested after 48-hour incubation. After trypsinization, cells were centrifuged at 314.5×G for 10 minutes, and washed in ice-cold PBS, then fixed overnight in 75%-ethanol and stored at -20°C until assayed. For analysis, stored samples were centrifuged at 314.5×G for 10 minutes and washed twice in PBS. The pelleted samples were re-suspended and incubated in propidium iodine (PI, Sigma) solution for 20 minutes at 4°C. RNase was added in the staining solution to avoid RNA contamination. The stained fibroblast population was gated with forward scatter (FSc) vs. side scatter (SSc) plot to display the relationship of cell size vs. granularity. Data were acquired by a FACScan flow cytometer (BD Biosciences, San Jose, CA), with cell counts obtained during 5 minutes of flow at 60 μl/min, to create a DNA content-frequency histogram and analyzed with Flowjo software (Tree Star Inc). A Dean-Jett-Fox model was then used to quantify cell-cycle phases, giving the percentages of cells in G0/G1, S and G2/M. The doublet problem was resolved by a doublet discrimination gate.

\(\text{KCNJ2 overexpression and dominant-negative constructs.}\)
Lentiviral constructs were used, carrying wild-type or dominant-negative KCNJ2 cDNAs or pWPI negative control expressing GFP only, as previously reported. Lentivirus preparation was performed as previously described. Control-dog left atrial canine fibroblasts were isolated and grown in T75 flasks. At near-confluence (3-4 day culture), fibroblasts were trypsinized, counted and plated in 12-well plates at 4.4x10⁴ cells/well. After 4-6 hours for recovery, cells were transduced with lentiviral vectors at 50 m.o.i. After overnight incubation (approximately 15 hours), cells were washed 3 times with 10%-FBS.
containing medium. After an additional 48 hours of culture, electrophysiological studies, Ca\textsuperscript{2+}-imaging or flow cytometry were performed.

**MicroRNA-26a knockdown.**
For overexpression, sense and antisense oligonucleotides were synthesized by Invitrogen, and the double-stranded RNA was created by annealing. For knockdown, the anti-miR-26a oligonucleotide (AMO-26a) with locked nucleic acid chemistry was synthesized by Exiqon. Scrambled oligonucleotides with locked nucleic acid were used as negative controls (AMO-NC). Dog left atrial fibroblasts in primary culture were transfected with AMO-26a (10 nmol/L) or AMO-NC (10 nmol/L) with Lipofectamine 2000 (Invitrogen). Control-dog left atrial canine fibroblasts were isolated and grown in T75 flasks. At near-confluence (3-4 day culture), fibroblasts were trypsinized, counted and plated in 12-well plates at 4.4×10\textsuperscript{4} cells/well. After 4-6 hours for recovery, cells were exposed to AMO-26a, AMO-NC or vehicle in Lipofectamine. After overnight incubation, cells were washed 3 times with 10%-FBS medium. After an additional 48 hours of transfection, cells were used for patch-clamp studies.

**Taqman Real-time Quantitative Reverse Transcription (RT)-Polymerase-Chain Reaction (PCR).**
Freshly isolated dog fibroblasts were resuspended in a lysis buffer and RNA was isolated with Nucleospin RNA II (Macherey Nagel), including a DNase treatment to prevent genomic contamination. Messenger RNAs were reverse-transcribed with the High-capacity Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed with TaqMan probes and primers from Applied Biosystems for housekeeping genes HPRT, β2-microglobulin and G6PD, as well as for KCNJ2, collagen-1 (Col-1a1), collagen-3 (Col-3a1), fibronectin-1 (Fn1), fibrillin-1 (FBN1), and α-smooth muscle actin (α-SMA). SyBr green primers were used to quantify KCNJ12 and KCNJ4. The geometric mean expression of HPRT, β2-microglobulin and G6PD was used for normalization. Quantitative PCR reactions were performed with Taqman Gene Expression Master Mix (Applied Biosystems). Reactions were run on a Stratagene MX3000. Relative gene-expression values were calculated by the 2^-ΔCt method.

**Immunostaining.**
Freshly-isolated and cultured fibroblasts were rinsed with PBS and fixed for 10 minutes with 1:1 acetone/methanol at -20°C, then cells were blocked for 1 hour with 5%-BSA at room temperature. The fibroblasts were incubated with mouse anti-α-SMA (1/500, Sigma), goat anti-vimentin (1/500, Santa Cruz), followed by donkey anti-mouse IgG-Alexa Fluor 555 (1/500, Invitrogen), donkey anti-rabbit IgG-Alexa Fluor 488 (1/500, Invitrogen) and TOPRO-3 iodide (1/1000, Invitrogen). Fluorescent images were obtained with an Olympus Fluoview FV1000 inverted confocal microscope.

**Western blots.**
Protein was extracted, quantified, and processed as we previously described.\textsuperscript{8} Freshly-isolated fibroblasts were lysed in detergent-based buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Nonidet P-40, 1% Triton X-100, 1 mmol/L NaF, 1 mmol/L Na\textsubscript{3}VO\textsubscript{4} and protease inhibitors). Protein samples were separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated with mouse anti-α-SMA (1/1000, Sigma), goat anti-vimentin (1/1000, Santa Cruz), anti-Kir2.1 (1:200, Neuromab), and GAPDH (1/10000, Fitzgerald) antibodies. Secondary antibodies conjugated to horseradish peroxidase were used for detection via chemiluminescence.

**Data analysis.**
Clampfit 9.2 (Axon), GraphPad Prism 4.0, and Origin 5.0 were used for data analysis. All data are expressed as mean±SEM. Multiple group statistical comparisons were obtained by two-way ANOVA and individual group-mean differences evaluated by Student’s t-tests with Bonferroni correction. A 2-tailed P<0.05 was considered statistically-significant.
RESULTS

Properties of the model.

CHF significantly increased right-atrial ERP at all BCLs (Supplemental Figure IA). Mean AF duration increased substantially in CHF dogs (Supplemental Figure IB). CHF reduced arterial pressures and increased filling pressures (Supplemental Figure IC-E). Atrial histopathology confirmed the presence of fibrosis in CHF-dogs (Supplemental Figure II), and qPCR confirmed enhanced extracellular-matrix gene expression (Supplemental Figure IIIA, 3B).

CHF-induced changes in fibroblast phenotype.

Bright field microscopic images of freshly-isolated fibroblasts from each group are shown in Figure 1A, with CHF-fibroblasts being systematically larger (Figure 1B-D). Immunofluorescence suggested that CHF-fibroblasts exhibited enhanced vimentin and α-SMA expression (Figure 1E), an impression confirmed by image quantification (Figure 1F-G). Western blot analysis further supported CHF-induced fibroblast α-SMA and vimentin upregulation (Figure 1H-I). Gene expression mRNA-levels of the ECM proteins Col-1a1, Col-3a1, Fn1 and FBN were all greater in freshly-isolated CHF-fibroblasts versus control (Supplemental Figure IIIA, 3B). These observations indicate that CHF alters atrial fibroblast phenotype.

Changes in IK1 and RMP.

Figures 2A and B show examples of IK1 recordings from freshly-isolated fibroblasts obtained with the ramp protocol shown in the inset. The current was strongly suppressed by 300 µmol/L Ba2+. Overall data (Figure 2C) indicate significantly larger IK1 in CHF. CHF significantly increased expression of KCNJ2 (Kir2.1) at both mRNA (by 52%) and protein (by 80%) levels (Figures 2D, 2E). KCNJ12 (Kir2.2) mRNA expression (Supplemental Figure IIIC) was 2 orders of magnitude less than that of KCNJ2 (Supplemental Figure IIDD), and was not affected by CHF. KCNJ4 (Kir2.3) was undetectable. CHF significantly increased RMP (from -43.2±0.8 mV, control, to -55.5±0.9 mV, P<0.001, Figure 2F). Ba2+ (300 µmol/L) significantly reduced RMP and greatly attenuated the RMP differences between control and CHF-fibroblasts, suggesting that they were due to IK1 upregulation in CHF (Figure 2F). Similar effects were seen with a 10-fold larger Ba2+ concentration (3 mmol/L, Figure 2G). Of note, despite the statistically significant reduction in RMP with Ba2+, the fibroblasts maintained a negative RMP, indicating a contribution from conductances other than IK1.

To further verify the role of IK1 in fibroblast RMP changes with CHF, we performed the studies shown in Supplemental Figure IV. Consistent with expected behavior, the current conductance (and particularly the inward current component) was greatly enhanced (Supplemental Figures IVA and B) by increasing extracellular K+-concentration ([K+]o) to 75 mmol/L (equimolar substitution for Na+). In addition to enhancing conductance (Supplemental Figures IVB and D), increased [K+]o shifted the reversal potential positively, from -53.4±3.7 mV and -64.3±3.8 mV with 5.4 mmol/L [K+]o in control and CHF respectively, to -14.9±1.1 mV (P<0.001) and -15.1±1.1 mV (P<0.001) for control and CHF in 75 mmol/L [K+]o. The RMP was significantly reduced by 75 mmol/L [K+]o for both control and CHF conditions (Supplemental Figures IVB and F), and elevating [K+]o largely eliminated the RMP differences between control and CHF, with values averaging -30.9±1.5mV and -32.5±1.6mV respectively in 75 mmol/L [K+]o (P=NS).
Changes in fibroblast Ca\textsuperscript{2+}-entry.

Figures 3A and B show store-dependent Ca\textsuperscript{2+}-entry data from a control and CHF fibroblast respectively. Cells in short-term (20-hour) culture were first exposed to nominally Ca\textsuperscript{2+}-free extracellular solution to deplete Ca\textsuperscript{2+}-stores, and then Ca\textsuperscript{2+}-entry was observed upon increasing \( [\text{Ca}^{2+}]_o \) to 10 mmol/L (Figures 3A and B). Resting \( [\text{Ca}^{2+}]_i \) (Figure 3C) and store-dependent Ca\textsuperscript{2+}-entry (Figure 3D) were greater in CHF cells. We were unable to use Ba\textsuperscript{2+} as a probe to inhibit I\textsubscript{K1} and study the role of I\textsubscript{K1}-differences in Ca\textsuperscript{2+}-entry, because Ba\textsuperscript{2+} interacts directly with Fura-2.\textsuperscript{11} However, increasing \( [\text{K}^+]_o \) to reduce RMP substantially suppressed store-dependent Ca\textsuperscript{2+}-entry under both control and CHF conditions, and greatly reduced the difference between control and CHF values (Figure 3D). These results suggest that RMP is a significant determinant of fibroblast Ca\textsuperscript{2+}-entry, and that RMP differences due to I\textsubscript{K1} remodeling may contribute to the increased Ca\textsuperscript{2+}-entry caused by CHF.

Fibroblast proliferation and differentiation.

To study CHF-induced changes in fibroblast proliferation and differentiation, along with the potential contribution of I\textsubscript{K1} remodeling, we had to perform experiments with short-term (3-day) cultured cells. We first verified that 3-day culture does not alter fibroblast I\textsubscript{K1} or RMP (Supplemental Figure V). We then collected fibroblasts for proliferation analysis by flow cytometry. Figures 4A and B show representative DNA content histograms and Dean-Jett-Fox model fitting of control and CHF atrial fibroblasts (G0: resting phase; G1 phase: increased size and ready for DNA synthesis; G2/M phase: cells with doubled DNA content in pre-mitotic and mitotic phase). CHF increased total cell count and cell content in G2/M phase (Figure 4C and D). Mean total cell counts are shown in Figure 4C and percentages in each phase in Figure 4D. CHF significantly increased the percentage of cells in G2/M phase (Figure 4D), indicating increased proliferation. Cultured CHF fibroblasts also showed properties indicating greater myofibroblast differentiation versus control, including altered cell morphology and greater expression of vimentin and \( \alpha \)-SMA protein (Supplemental Figure VI).

I\textsubscript{K1} regulates RMP, Ca\textsuperscript{2+}-entry and proliferation.

The experiments shown in Figure 3 suggest that I\textsubscript{K1} may contribute to the control of atrial fibroblast Ca\textsuperscript{2+}-entry. To explore the functional contributions of I\textsubscript{K1} more directly, we used a gene-transfer approach to vary the current in fibroblasts. A KCNJ2 dominant-negative construct (KCNJ2-DN) with the GYG motif replaced by a triple-alanine (AAA) sequence was used to suppress endogenous KCNJ2 current. Wild type KCNJ2 overexpression (KCNJ2-OE) was used to enhance the current. KCNJ2-DN and wild type KCNJ2 were packed into a lentivirus vector containing GFP. A control virus was also prepared that contained only GFP inserted into the lentiviral vector. Infected cells were identified by green fluorescence.

Figure 5A shows original recordings of I\textsubscript{K1} in fibroblasts infected with control (lentivirus carrying GFP alone), KCNJ2-DN, and KCNJ2-OE constructs, before and after the addition of 300 \( \mu \text{mol/L} \) Ba\textsuperscript{2+} to the superfusate. Currents were reduced by KCNJ2-DN and increased by KCNJ2-OE, and strongly suppressed by Ba\textsuperscript{2+}. Figure 5B shows current-voltage relations, with an inset showing control and KCNJ2-DN currents on an expanded current scale for clearer resolution. KCNJ2-OE greatly increased I\textsubscript{K1}, whereas KCNJ2-DN strongly reduced I\textsubscript{K1} density. Compatible with a role of I\textsubscript{K1} in FB differentiation, cell capacitance increased with KCNJ2-OE (Figure 5C). KCNJ2-OE substantially hyperpolarized the RMP, and consistent with the effects of Ba\textsuperscript{2+} shown in Figure 2F-G, KCNJ2-DN significantly reduced RMP. Exposure to Ba\textsuperscript{2+} eliminated the RMP differences among constructs (Figures 5D and E) with full effects seen at 300 \( \mu \text{mol/L} \), confirming that they are due to I\textsubscript{K1}.

We then went on to use the gene transfer approach to confirm directly the ability of I\textsubscript{K1} to regulate atrial fibroblast Ca\textsuperscript{2+}-entry and proliferation. KCNJ2-DN decreased, and KCNJ2-OE increased, resting
Ca$^{2+}$-level (Figure 6A) and store-operated Ca$^{2+}$-entry (Figure 6B). Increased [K$^+$]o attenuated Ca$^{2+}$-entry in the presence of the lentivirus-GFP control vector and KCNJ2-OE (Figure 6B), but as expected given the virtual elimination of I$_{K1}$ produced by KCNJ2-DN, elevated [K$^+$]o did not alter Ca$^{2+}$-entry in the presence of KCNJ2-KD. Fibroblast proliferation indices were enhanced by KCNJ2-OE and suppressed by KCNJ2-DN (Figures 6C-D). These results confirm the role of I$_{K1}$ in governing fibroblast Ca$^{2+}$-entry and proliferation.

Role of RMP in controlling fibroblast Ca$^{2+}$-entry.

The most obvious way in which changes in I$_{K1}$ could affect fibroblast Ca$^{2+}$-entry is through resulting changes in RMP and the voltage gradient driving Ca$^{2+}$ into the cell. To test directly the effect of RMP on fibroblast Ca$^{2+}$-entry, we studied store-operated Ca$^{2+}$-entry in fibroblasts under voltage clamp conditions, with voltages set to approximate the RMP of control fibroblasts (-40 mV) and CHF fibroblasts (-55 mV). Figure 7A shows [Ca$^{2+}$]$_i$ recordings in one cell while being held at -40 mV (left panel) and then in the same cell at -55 mV (right). Hyperpolarizing the fibroblast increased the Ca$^{2+}$-transient amplitude. Figure 7B shows the mean Ca$^{2+}$-transient amplitude in cells in which we were able to study Ca$^{2+}$-entry under stable condition under both voltages (order randomized in different cells). RMP had a highly significant effect on Ca$^{2+}$-transient amplitude.

MicroRNA-26 regulation of atrial fibroblast I$_{K1}$.

The larger increase in KCNJ2 protein than mRNA in CHF points to mediation by microRNA. We have previously shown that miR-26 targets I$_{K1}$, and that its downregulation in cardiomyocytes from animals with sustained-AF governs cardiomyocyte I$_{K1}$-enhancement.12 We therefore considered the possibility that miR-26 regulation may contribute to the atrial-fibroblast I$_{K1}$-enhancement that we observed in CHF. Expression of the miR-26a isoform was decreased in freshly-isolated left atrial fibroblasts from CHF-dogs, while miR-26b was unaffected (Figure 8A). AMO-26a transfection into atrial fibroblasts with lipofectamine effectively suppressed miR-26a (Figure 8B, left). To exclude nonspecific effects, we examined miR-21 expression, which was unaffected by AMO-26a (Figure 8B, right). We then looked at the result of knocking down miR-26a, to mimic its downregulation in CHF, on I$_{K1}$ in atrial fibroblasts. Figure 8C shows original recordings from a fibroblast exposed to lipofectamine alone, a fibroblast exposed to a scrambled-control oligonucleotide (AMO-NC) and a fibroblast transfected with AMO-26a. I$_{K1}$ was clearly larger after AMO-26a exposure, as indicated by the mean current-voltage data in Figure 8D. Finally, we examined the effect of miR-26a knockdown on atrial-fibroblast RMP, and noted substantial hyperpolarization (Figure 8E).

DISCUSSION

In the present study, we analyzed the consequences of CHF-induced I$_{K1}$ upregulation in fibroblasts on fibroblast function, noting hyperpolarized RMP, enhanced Ca$^{2+}$-entry and increased proliferation indices. The mechanistic role of I$_{K1}$ changes was supported by genetically modifying I$_{K1}$ through KCNJ2 overexpression and knockdown, and the potential contribution of hyperpolarization to CHF-induced fibroblast Ca$^{2+}$-entry increases was demonstrated by simultaneous voltage-clamp and Ca$^{2+}$ microfluorometry. CHF-induced miR-26a downregulation was implicated as the mechanism of KCNJ2/I$_{K1}$ upregulation.
Functional role of ion channels in cardiac fibroblasts.

Although the presence of ion channels in cardiac fibroblasts is well established, their functional role is less clear. Ca²⁺-entry via nonselective cation channels of the transient receptor potential (TRP) family plays a role in fibroblast proliferation, differentiation and ECM-protein secretion. There is evidence that this action is mediated via Ca²⁺-dependent activation of extracellular signal-related protein-kinases (ERKs) and contributes to the AF-related arrhythmogenic substrate. The function of fibroblast K⁺-channels is less clear. Previous work has indicated that Kir channels contribute to RMP-determination. In cell co-culture systems, myofibroblasts can be shown to couple to cardiomyocytes and alter their electrophysiological properties, inducing a variety of arrhythmogenic mechanisms. While the importance of fibroblast-cardiomyocyte coupling in vivo is still controversial, mathematical modeling work suggests that it may account for complex fractionated electrogram properties in fibrotic tissues. We have recently evaluated the effects of fibroblast ion channel remodeling on the potential electrical and arrhythmogenic interactions between coupled fibroblasts and cardiomyocytes, finding that if fibroblasts were well coupled to cardiomyocytes, fibroblast Kv-current downregulation would suppress the AF-substrate whereas Kir-current upregulation enhance it. We have also obtained evidence for a profibrotic role of Kv-current downregulation, although the underlying mechanism is unclear.

Control of fibroblast Ca²⁺-entry and function by Kir-currents.

The present study is the first of which we are aware to show the control of fibroblast Ca²⁺-entry and proliferation by Kir2.1 current. A number of lines of evidence converged to clarify the role of fibroblast IK1. IK1 block with Ba²⁺ or K⁺ driving-force reduction by elevating [K⁺]o reduced the RMP, and elevated [K⁺]o reduced fibroblast store-operated Ca²⁺-entry. Functional KCNJ2 knockdown with dominant-negative overexpression reduced atrial fibroblast IK1, RMP, Ca²⁺-influx and proliferative activity, whereas KCNJ2 overexpression had the opposite effects. The role of RMP in mediating IK1 effects on Ca²⁺-entry was directly supported by experiments showing that hyperpolarization of voltage-clamped fibroblasts enhanced fibroblast store-operated Ca²⁺-entry.

Although this functional role has never before been described in fibroblasts, there is supportive evidence from prior work in endothelial cells. Bradykinin-induced changes in bovine endothelial cell cytosolic Ca²⁺ are consistent with an influx mechanism directly related to the Ca²⁺ electrochemical gradient. Nitric oxide synthesis and proliferation of umbilical-cord endothelial cells induced by basic fibroblast growth factor appears to depend on inward-rectifier K⁺ current augmentation. Finally, hyperpolarization increases cytoplasmic [Ca²+] in arteriolar endothelial cells.

Novel elements and potential significance.

Cardiac fibrosis is an important contributor to cardiac dysfunction and arrhythmogenesis, and is a particularly significant contributor to the substrate that allows enhanced AF maintenance in CHF. Fibroblasts play a central role in the fibrotic process. Here, we addressed a novel regulatory aspect of fibroblast physiology, functional control by IK1, along with the remodeling of IK1 and its contribution to altered fibroblast function in a clinically relevant fibrotic paradigm: the CHF-induced atrial-profibrillatory substrate. We report for the first time that CHF-related atrial IK1-upregulation and consequent fibroblast-hyperpolarization enhance fibroblast Ca²⁺-entry and cell proliferation. This work identifies a novel participant in the profibrotic response, with potential implications for the development of novel therapeutic interventions. Ion channels are targets for new antiarrhythmic agents. Our study shows that in addition to altering cardiac electrophysiology, interventions that target IK1 may affect cardiac structural remodeling, particularly since the principal IK1-subunit, KCNJ2/Kir2.1, is common to both cardiomyocytes and fibroblasts. The predominance of KCNJ2 in cardiac IK1 is well recognized. The present study indicates that KCNJ2 is similarly predominant in fibroblasts: DN-KCNJ2 almost
completely eliminated fibroblast \( I_{K1} \) (Figure 5B inset). \( I_{K1} \) blockers are being developed as potential antiarrhythmic molecules,\(^{25,26}\) based on their ability to inhibit cardiomyocyte \( I_{K1} \) and destabilize AF-maintaining rotors.\(^{26,27}\) An additional potentially interesting consequence, based on the work reported here, might be suppression of atrial fibrosis. The converse may also hold. Fibroblasts engineered to overexpress Kir2.1, Nav1.5 and connexin-43 subunits rescue normal propagation and decrease arrhythmia complexity in co-cultured cardiomyocyte-fibroblast monolayers.\(^{28}\) A risk of applying this approach therapeutically might be profibrotic consequences of increased \( I_{K1} \).

MicroRNAs are significant control-molecules in cardiac remodeling.\(^{29}\) We have previously shown that miR-26 downregulation contributes to AF-promoting remodeling by upregulating cardiomyocyte \( I_{K1}.\)\(^{12}\) Here, we have identified an additional profibrillatory consequence of disease related miR-26 downregulation: fibroblast activation via fibroblast \( I_{K1} \)-upregulation consequent to removal of miR-26 induced negative regulation of the KCNJ2 gene.

**Potential limitations.**

Fibroblast hyperpolarization significantly increased store-operated \( Ca^{2+} \)-entry by about 30% (Figure 7B); however, CHF fibroblasts showed an approximately 70% increase in \( Ca^{2+} \)-entry (Figure 3D). Thus, the hyperpolarization caused by \( I_{K1} \) upregulation is likely not the only factor increasing \( Ca^{2+} \)-entry in CHF fibroblasts. In addition to KCNJ2, miR-26 controls expression of the gene encoding TRPC3 subunits.\(^{8}\) TRPC3 subunit upregulation due to miR-26 downregulation in AF enhances fibroblast \( Ca^{2+} \)-entry.\(^{8}\) Thus, TRPC3 expression changes caused by CHF-induced miR-26a downregulation likely also contributed to the increased fibroblast \( Ca^{2+} \)-entry observed in CHF fibroblasts in the present study.

We performed experiments in isolated fibroblasts in order to evaluate their cell biology in detail. Analysis of fibroblast properties in situ is greatly complicated by their very small size and a dearth of specific probes. Paracrine effects in vivo could significantly alter fibroblast behavior and was not analyzed here. In addition, in this study we measured fibrous tissue content only in the left atrial appendage (Supplemental Figure II). The distribution of fibrosis may not be uniform in atria. Fibroblast proliferation might be affected by lentivirus infection. We therefore verified cell counts upon culture in control fibroblasts versus lentivirus-GFP infected fibroblasts and found no significant differences (Supplemental Figure VII).

We used \( Ba^{2+} \) as one of several tools to compare the contribution of \( I_{K1} \) in CHF fibroblasts to that in control. \( Ba^{2+} \) can affect a variety of \( K^{+} \)-currents, and the possibility of nonspecific effects requires caution in the interpretation of data. Evidence against any significant nonspecific effects of \( Ba^{2+} \) in our system is provided by the results of KCNJ2 knockdown on the response to \( Ba^{2+} \) (Supplemental Figure VIII). \( Ba^{2+} \) had no significant effect on currents once KCNJ2/\( I_{K1} \) was knocked down, indicating the absence of any significant effect on other currents under our recording conditions.
ACKNOWLEDGMENTS
The authors thank Nathalie L’Heureux, Chantal St-Cyr and Audrey Bernard for technical assistance, and France Thériault for secretarial help with the manuscript.

SOURCES OF FUNDING
This work was supported by the Canadian Institutes of Health Research (44365, 6957), the Heart and Stroke Foundation of Canada and the Fondation Leducq (European-North American Atrial Fibrillation Research Alliance; 07CVD03).

DISCLOSURES
None.

REFERENCES


FIGURE LEGENDS

**Figure 1.** A, Microscopic images of freshly-isolated fibroblasts from control (CTL) and CHF canine left atria. B-D, Mean±SEM cell diameter, cell area and cell membrane capacitance of CTL and CHF fibroblasts. E, Immunofluorescent images of freshly-isolated CTL and CHF fibroblasts. Staining shown is vimentin (green) and α-SMA (red). F-G, Mean±SEM α-SMA and vimentin immunofluorescence-quantification based on images like those in E. H-I, Mean±SEM α-SMA and vimentin immunoblots band intensities/GAPDH band intensities. *P<0.05, **P<0.01, ***P<0.001 CTL vs CHF; n/N=cells/dogs per group.

**Figure 2.** A-B, I_{K1} recordings (at 0.1 Hz) from CTL and CHF freshly-isolated atrial fibroblasts. C, Mean±SEM current density versus voltage relations for Ba^{2+}-sensitive I_{K1} in freshly-isolated atrial fibroblasts. D, Mean±SEM KCNJ2 mRNA-expression. E, Mean±SEM Kir2.1 protein-expression. F-G, Mean±SEM α-SMA and vimentin immunofluorescence-quantification based on images like those in E. H-I, Mean±SEM α-SMA and vimentin immunoblots band intensities/GAPDH band intensities. *P<0.05, **P<0.01, ***P<0.001 CTL vs CHF; n/N=cells/dogs per group.

**Figure 3.** A, B, Recordings of store-operated Ca^{2+}-influx in CTL and CHF freshly-isolated atrial fibroblasts, in the presence of 5.4 mmol/L or 75 mmol/L [K^+]_o, with protocol shown at top. C, Mean±SEM resting intracellular [Ca^{2+}]. D, Mean±SEM store-dependent Ca^{2+}-influx. *P<0.05, **P<0.01, CTL vs CHF; *P<0.05, 5.4 mmol/L [K^+]_o vs 75 mmol/L [K^+]_o; n/N=cells/dogs per group.

**Figure 4.** A-B, Representative DNA-content histograms and Dean-Jett-Fox model fitting of 3-day cultured atrial fibroblasts from a control (CTL) and a CHF dog. C, Mean±SEM cell count of atrial fibroblasts from CTL and CHF dogs. D, Mean±SEM percentage of cells in G0/G1 and G2/M phases. *P<0.05, **P<0.01, ***P<0.001 CTL vs CHF.

**Figure 5.** A, Representative I_{K1} recordings in GFP-expressing control (Lenti-GFP), dominant negative KCNJ2 (KCNJ2-DN) and KCNJ2-overexpressing (KCNJ2-OE) fibroblasts in primary culture. B, Mean±SEM Ba^{2+}-sensitive I_{K1} density-voltage relations in cells infected with Lenti-GFP, KCNJ2-DN and KCNJ2-OE vectors. Inset: Lenti-GFP and KCNJ2-DN data on enlarged current scale. C, Mean±SEM cell capacitance. D-E, Mean±SEM RMP before and after 300 µmol/L and 3 mmol/L Ba^{2+}. **P<0.01, ***P<0.001 CTL vs Lenti-GFP. *P<0.05, **P<0.01 pre-Ba^{2+} vs post-Ba^{2+}; n/N=cells/dogs per group.

**Figure 6.** A, Mean±SEM resting intracellular [Ca^{2+}] in GFP-expressing control (Lenti-GFP), KCNJ2 dominant negative (KCNJ2-DN) and KCNJ2 overexpressing (KCNJ2-OE) fibroblasts. B, Mean±SEM Ca^{2+}-influx (Δ ratio) at 5.4 and 75 mmol/L [K^+]_o. C, Mean±SEM cell count of atrial fibroblasts in various phases from Lenti-GFP, KCNJ2-DN and KCNJ2-OE groups. D, Mean±SEM percentage of cells in G0/G1 and G2/M phases. *P<0.05, ***P<0.001 vs Lenti-GFP; *P<0.05, **P<0.01, 5.4 vs 75 mmol/L [K^+]_o; n/N=cells/dogs per group.

**Figure 7.** A. Original recordings of intracellular [Ca^{2+}] in one fibroblast held at -40 mV (left) and -55 mV (right) to mimic RMP in control and CHF fibroblasts. B, Mean±SEM Ca^{2+}-influx (Δ ratio) in 24 cells held at both -40 and -55 mV.

**Figure 8.** A, Mean±SEM relative microRNA expression in freshly-isolated left atrial fibroblasts. B, Efficiency of miR-26a knockdown by anti-miR-26a oligonucleotide (AMO-26a) in canine left atrial fibroblasts. Lipo: lipofectamine control; AMO-NC: scrambled oligonucleotides with methylene bridges used as negative control for AMO-26a. miR-21 was unaffected by AMO-26a. C, I_{K1} recordings (voltage ramp protocol in inset delivered at 0.1 Hz). D, Mean±SEM 300 µM Ba^{2+}-sensitive I_{K1} density. E, Mean±SEM RMP. **P<0.01, ***P<0.001 Lipo vs AMO-26a; n/N=cells/dogs per group.
Novelty and Significance

What Is Known?

- Cardiac fibroblasts play a central role in tissue fibrosis, which is an important contributor to a variety of arrhythmias, including atrial fibrillation (AF).
- Cardiac fibroblasts possess a range of ion channels, but the functional role of fibroblast ion channels is poorly understood.
- Congestive heart failure (CHF) causes prominent atrial fibrosis, and is therefore a major risk factor for the arrhythmia.

What New Information Does This Article Contribute?

- Congestive heart failure leads to the upregulation of the background inward rectifier potassium current ($I_{K1}$) in atrial fibroblasts, likely by downregulating a microRNA (miR-26) that targets $I_{K1}$.
- An increase in atrial fibroblast $I_{K1}$ hyperpolarizes the cell membrane, enhancing $Ca^{2+}$ entry by increasing the driving force for transmembrane $Ca^{2+}$ movement.
- These findings define a new pathway for CHF-induced atrial fibrosis involving an increase in $I_{K1}$, leading to hyperpolarization and enhanced calcium entry, and resulting in fibroblast activation.

CHF an important clinical risk factor for AF, the commonest sustained arrhythmia and a major source of morbidity and mortality. While CHF-induced atrial fibrosis is believed contribute to the AF substrate, the mechanisms leading to this fibrosis are poorly understood. Moreover, the functional roles of ion-channels in fibroblasts, the cells that produce fibrosis, are not well understood. Here, we studied changes in the background inward rectifier potassium current ($I_{K1}$) in atrial fibroblasts from dogs with CHF induced by ventricular tachypacing. We found significant upregulation of $I_{K1}$, which hyperpolarized the resting membrane potential of the fibroblasts. This hyperpolarization enhanced $Ca^{2+}$-entry, known to be an important fibroblast activating mechanism, causing fibroblasts to proliferate. We also found that $I_{K1}$ upregulation is caused by CHF-induced decreases in atrial fibroblast expression of a microRNA, miR-26, that targets the gene ($KCNJ2$) encoding $I_{K1}$. These findings reveal a new function of fibroblast potassium channels, i.e., the control of fibroblast activation, and show that these channels can mediate a pathological arrhythmia-promoting response. Further elucidation of this signaling pathway would provide novel insights into the mechanisms controlling AF and might allow for the development of new therapeutic approaches.
Figure 1

A  
CTL  
CHF  

20 μm  

B  
Cell diameter (μm)  

CTL  
CHF  
45/4  
44/3  

C  
Cell area (μm²)  

CTL  
CHF  
75/4  
105/3  

D  
Cm (pF)  

CTL  
CHF  
117/10  
98/10  

E  
Vimentin  
αSMA  

20 μM  

F  
αSMA (A.U.)  

CTL  
CHF  
75/5  
105/5  

G  
Vimentin (A.U.)  

CTL  
CHF  
75/5  
105/5  

H  
α-SMA/GAPDH  

CTL  
CHF  
N=8  
N=11  

I  
Vimentin/GAPDH  

CTL  
CHF  
N=4  
N=4  

***  
*  

Downloaded from http://circres.ahajournals.org/ on July 1, 2017.
Figure 4

A

![CTL Cell Count Graph](image1)

B

![CHF Cell Count Graph](image2)

C

![Cell Count Bar Graph](image3)

D

![Cell Percentage Bar Graph](image4)
Figure 6

A

Ca\(^{2+}\) Ratio F340/F380

Resting Ca\(^{2+}\)

Lenti-GFP  
KCNJ2-DN  
KCNJ2-OE

n/N=13-15/7

***


B

Δ Ca\(^{2+}\) Ratio F340/F380

mM K\(^{+}\)

5.4 75 5.4 75 5.4 75

Lenti-GFP  
KCNJ2-DN  
KCNJ2-OE

n/N=13-15/7

***

***

###

C

Cell Number x 10^4

Total  
G0/G1  
G2/M

N=6

***

***

*


D

Cell percentage (%)

G0/G1  
G2/M

N=6

*

***
Figure 7

A

\[ \text{Ca}^{2+} \text{ Ratio F400/F500} \]

\[ \text{Time (s)} \]

B

\[ \Delta \text{Ca}^{2+} \text{ Ratio F400/F500} \]

-40 mV  n/N=24/3

-55 mV  n/N=24/3

***
Fibroblast Inward-Rectifier Potassium Current Upregulation in Profibrillatory Atrial Remodeling

Xiao-Yan Qi, Hai Huang, Balázs Ördög, Xiaobin Luo, Patrice Naud, Yiguo Sun, Chia Tung Wu, Kristin Dawson, Artavazd Tadevosyan, Yu Chen, Masahide Harada, Dobromir Dobrev and Stanley Nattel

_Circ Res._ published online January 21, 2015;

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2015 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/early/2015/01/21/CIRCRESAHA.116.305326

Data Supplement (unedited) at:

http://circres.ahajournals.org/content/suppl/2015/01/21/CIRCRESAHA.116.305326.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:

http://circres.ahajournals.org/subscriptions/
Supplemental Material

Fibroblast Inward-Rectifier Potassium Current Upregulation in Profibrillatory Atrial Remodeling

Xiao-Yan Qi, PhD,* Hai Huang, PhD,* Balazs Ordog, PhD, Xiaobin Luo, PhD, Patrice Naud, PhD, Yiguo Sun, PhD, Chia-Tung Wu, MD, Kristin Dawson, PhD, Artavazd Tadevosyan, PhD, Yu Chen, MSc, Masahide Harada, MD, PhD, Dobromir Dobre, MD, Stanley Nattel, MD

* Both authors contributed equally

Supplemental Figures
Supplemental Figure 1

A, Mean±SEM atrial effective refractory period (ERP) as a function of basic cycle length (BCL).

B, Mean±SEM duration of induced atrial fibrillation (AF) in control (CTL) and CHF.

C, Mean±SEM blood pressure (BP).

D, Mean±SEM left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP).

E, Mean±SEM left atrial pressure (LAP) and right atrial pressure (RAP).

*P<0.05, **P<0.01, ***P<0.001 vs CTL.
Supplemental Figure II. A-B, Masson's trichrome-stained light micrographs from a CTL (A) and a CHF (B) dog left atrial appendage (×200 magnification). C, Mean±SEM fibrosis tissue content (percentage of cross-sectional area) in left atrial appendage ***P<0.001 vs CTL.
Supplemental Figure III. A-B. Mean±SEM extracellular matrix gene mRNA-expression in freshly-isolated atrial fibroblasts from CTL and CHF dogs. C, Mean±SEM KCNJ12 (Kir2.2) mRNA expression level. D, Mean±SEM KCNJ2 (Kir2.1) mRNA expression level. *P<0.05, **P<0.01, ***P<0.001 CTL vs CHF.
Supplemental Figure IV. A-B. Inwardly-rectifying K\(^+\)-current inward components are enhanced by 75 mol/L [K\(^+\)]\(_o\) (reversal potential also shifted in the positive direction) and currents are blocked by 300 µmol/L Ba\(^{2+}\) in freshly-isolated CTL and CHF atrial fibroblasts. C-D, Mean±SEM inwardly rectifying K\(^+\) current-density. E-F, Mean±SEM RMP in CTL and CHF freshly-isolated atrial fibroblasts before and after 75 mmol/L [K\(^+\)]\(_o\)-exposure. *, **, ***: P<0.05, 0.01, 0.001 for 5.4 mmol/L K\(^+\) vs 75 mmol/L K\(^+\), n/N=cells/dogs per group.
Supplemental Figure V. A, Mean±SEM current-voltage relations of Ba^{2+}-sensitive current from freshly-isolated and 3-day cultured atrial fibroblasts. B, Mean±SEM RMP from freshly-isolated and 3-day cultured atrial fibroblasts.
Supplemental Figure VI. A, Immunofluorescent images of 3-day cultured control (CTL) dog atrial fibroblasts. B, Immunofluorescent images of 3-day cultured CHF dog atrial fibroblasts. Staining shown is vimentin (green), α-SMA (red), TOPRO3 (blue, nuclear) and merged.
Supplemental Figure VII. Comparison of fibroblast proliferation in A, Control (CTL) fibroblasts and B, Lenti-GFP infected fibroblasts. Bright-field images are at left; fluorescent images are at the right. Cell counts after 3 days in culture were 58.2 ± 4.5/high-power field (HPF) for CTL versus 57.0 ± 3.2/HPF for Lenti-GFP (N=5, 6 respectively). To obtain cell counts, cells were cultured in the same general way as for flow cytometry, but rather than being passed through a flow cytometer, cells were counted visually under microscopy with a 40× objective lens.
Supplemental Figure VIII. Currents measured in cells infected with KCNJ2-DN bearing lentivirus, before and after exposure to 300 µmol/L Ba^{2+}. Ba^{2+} had no statistically significant effect on these currents (2-way ANOVA, Ba^{2+} effect F=0.27, P=0.606).