SUPPLEMENT MATERIAL

TRPC3-dependent Fibroblast Regulation in Atrial Fibrillation

Supplemental Methods

Rat fibroblast isolation and culture

Adult male Sprague-Dawley rats weighing 200-250 g were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Hearts were quickly excised via thoracotomy into ice-cold Tyrode solution containing (mol/L) 140 NaCl, 5.4 KCL, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, and 5.5 glucose (pH 7.35 with NaOH) and Langendorff-perfused at 37°C with 1) Tyrode solution for 5 min; 2) Ca^{2+}-free Tyrode solution for 5 min; 3) Ca^{2+}-free Tyrode solution containing 0.04 mg/ml collagenase-II for 30 min. They were then removed, minced, and homogenized in Ca^{2+}-free Tyrode solution. Isolated cells were centrifuged (550 rpm, 3 min), separating cardiomyocytes (primarily in the pellets) from fibroblasts (in the supernatant). Cardiomyocytes were further removed by passing the supernatant through a 20-µm nylon filter; fibroblasts were then further concentrated via centrifugation (2000 rpm, 10 min). Pellets were resuspended and washed twice in M199-medium supplemented with 10% fetal bovine serum for primary culture.

Atrial tissue-samples from humans, goats, and dogs

Human right-atrial appendage biopsies were obtained from patients in sinus rhythm and with chronic AF during coronary artery bypass graft surgery. The study was approved by the ethical review committee of Dresden University of Technology. All subjects gave informed consent. AF was induced in chronically-instrumented goats using repetitive burst-pacing for 10 days. Congestive heart failure

(CHF) dogs with AF substrates were created by rapid ventricular pacing (240-bpm, 2 weeks). Normal goats and dogs were used as controls. Right-atrial tissue samples were collected and fast-frozen in liquid-N_2.

AF-Dogs

A total of 48 mongrel dogs (20-36 kg) were divided into control and atrial-tachypacing groups. Animal-care procedures followed National Institutes of Health guidelines (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. Dogs were anesthetized with ketamine (5.3 mg/kg i.v.), diazepam (0.25 mg/kg, i.v.), and isoflurane (1.5%), intubated, and ventilated. A unipolar pacing lead was inserted into the right-atrial appendage under fluoroscopic guidance. The lead was connected to a pacemaker (Star Medical, Tokyo, Japan) implanted in the neck. Two bipolar electrodes were inserted into the right-ventricular apex and right-atrial appendage for internal electrocardiogram (ECG) recording. Atrioventricular-block and ventricular pacing as employed in many studies of atrial-tachycardia remodeling[1] was not performed, to more closely mimic spontaneous clinical AF-episodes. The atrial pacemaker was programmed to stimulate the right-atria at 600-bpm for 1 week, with fibrillatory atrial activity maintained during pacing as assessed by daily ECG and intracardiac recordings. Echocardiography was performed on Day 0 (before atrial pacing, baseline) and Day 7 to assess changes in LA dimension, LA systolic function and LV diastolic volume.

For in vivo treatment of the AF dogs, an Alzet osmotic pump (model 2ML1) was implanted subcutaneously in the back and a selective TRPC3 blocker, pyrazole3 (0.1 mg/kg/day, dissolved in DMSO and polyethylene glycol), or vehicle (DMSO and polyethylene glycol) was continuously administrated for the entire period of atrial tachypacing (Supplemental Figure 3).

Dog atrial fibroblast isolation and culture

After the open chest study, the left-atrial tissues were immersed in oxygen-saturated, Ca^{2+}-containing Tyrode solution at room temperature. The left circumflex coronary artery was cannulated and perfused with Ca^{2+}-containing Tyrode-solution (37°C, 10 min), then perfused with Ca^{2+}-free Tyrode-solution (15 min), followed by 50-minute perfusion with Tyrode-solution containing collagenase (Worthington, type II) and 1% bovine serum albumin (Sigma). Digested left-atrial tissue was then removed, minced, and homogenized in M199 media. Isolated cells were centrifuged (800 rpm, 3 min) to separate cardiomyocytes (primarily in the pellets) from fibroblasts (in supernatant). Cardiomyocytes were further removed by filtration of the supernatant through a 20-µm nylon filter; fibroblasts were then further concentrated via centrifugation (2000 rpm, 10 min). Pellets were resuspended and washed twice in M199-medium supplemented with 10% fetal bovine serum for primary culture.

Terminal Open-chest Electrophysiological Study

Dogs were anesthetized with morphine (2 mg/kg, s.c.) and α-chloralose (120 mg/kg, i.v., followed by 29.25 mg/kg/h), and mechanically ventilated. Body temperature was maintained at 37°C. A median sternotomy was performed, and bipolar electrodes were hooked into the RA appendages for recording and stimulation. Right-atrial effective refractory period (ERP) was measured at basic cycle lengths of 150, 200, 250, 300, and 360 ms with 10 basic (S1) stimuli, followed by an S2 with 5-ms decrements (all pulses twice-threshold, 2-ms). AF (irregular atrial rhythm > 400 bpm) was induced with 1-10 s atrial burst-pacing (10-20 Hz, 4×threshold, 2-ms pulses). Mean AF-duration was determined in each dog based on 10 AF-inductions for AF<5 min and 5 inductions for 5-30 min AF. AF>30 min was considered sustained: cardioversion was not performed, and electrophysiological assessment was terminated. Haemodynamic data were obtained with fluid-filled catheters and transducers.

qPCR

Total RNA and microRNA were extracted with TRIzol (Invitrogen) and mirVana miRNA Extraction Kit (Ambion) from freshly isolated fibroblasts, respectively. Real-time RT-PCR was performed with 6-carboxy-fluorescein (FAM)-labeled fluorogenic Taq-Man primers and probes (Applied Biosystem). Fluorescence-signals were detected in duplicate, normalized to β2-microglobulin RNA for total RNA and to U6 snRNA for microRNA, and quantified with MxPro qPCR-software (Stratagene).

TaqMan low-density arrays

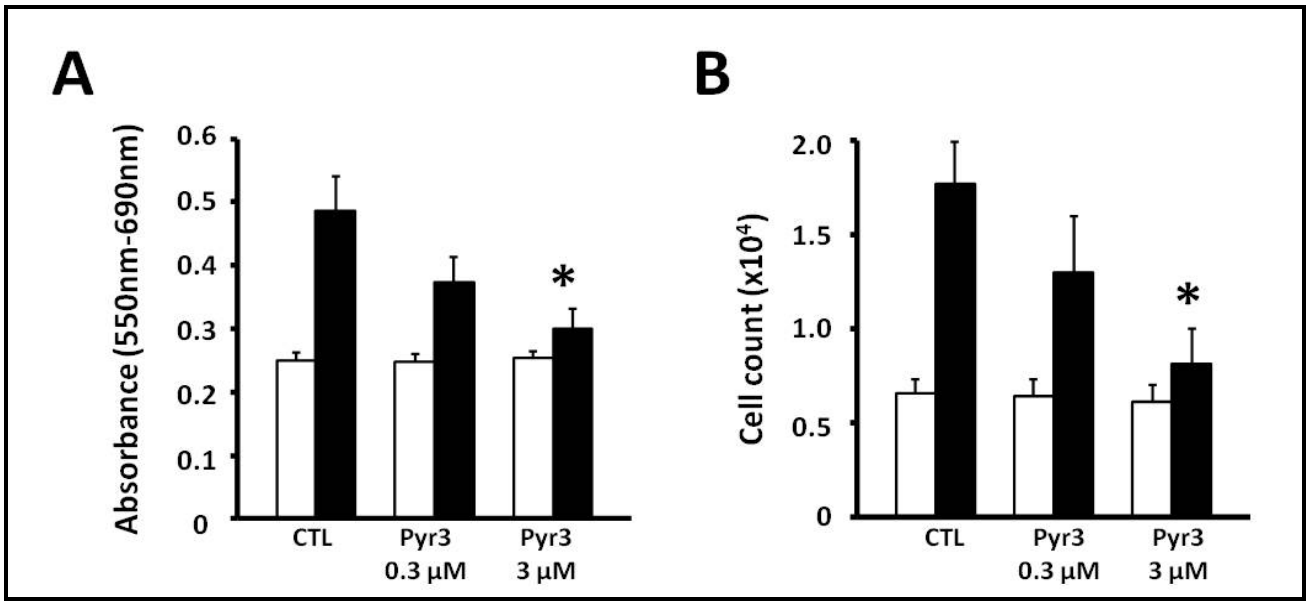
Total RNA was extracted using TRIzol. RNA-integrity was assessed via Agilent Bioanalyzer. (RIN>7.5 required). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random hexamer primers. TaqMan low-density arrays (TLDA, Applied Biosystems) were used in two-step RT-PCR as previously reported.[2] Real-time RT-PCR was performed on the 7900HT Fast Real-Time PCR System. Data were collected with SDS2.3 software and grouped with RQ Manager software. TRP-subunit genes were investigated using inventoried Taqman assays. Ct-methodology was applied to determine mRNA expression-levels. The mean expression of the genes with a Ct value below 30 (Ct<30) was selected as the reference.[3]

Cell-Proliferation and Cell-Cycle Analysis

Isolated cardiac fibroblasts were counted with hematocytometer and placed into T25 culture-flasks (2.0×10^5 cells/flask, 25 cm^2 growth-area) for each treatment group. Treatment was performed on Day 2 or Day 3 unless otherwise specified. After 1-hour and 24-hour incubation, cultured fibroblasts were totally harvested from each flask following trypsinization, then washed in ice-cold PBS and fixed in

1.0 ml of ice-cold 75% ethanol. Samples were stored at -20°C until analysis. Once pelleted, fibroblasts were re-suspended and incubated (4°C, 30 minutes) in 250 µl of staining solution containing propridium iodide (PI, Sigma) with RNAase and then 750 µl of PBS was added; the final volume in each sample was 1000 µl. In each preparation, the numbers of cells and PI fluorescence were measured with a FACScan (constant flow-rate, 60 µl/min, 5-min acquisition time, BD Bioscience) at 617-nm emission-wavelength to create a DNA content-frequency histogram. Samples were gated on fibroblast population using forward scatter vs. side scatter plot (cell size vs. granularity) and doublet discrimination gating. The percentage of cells in each phase of the cell cycle, G0/G1, S and G2/M phases, was analyzed using Flowjo software Dean-Jett-Fox model that fits G1 and G2 with Gaussian curves automatically (Tree Star Inc.).[4]

In order to confirm the accuracy of cell-counting with flowcytometry, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation assay was also performed with using Cell Proliferation Kit I (Roche). Two-day cultured fibroblasts were resuspended and transferred into a 96-well plate (5.0×10^3 cells/well, 0.32 cm^2 growth-area). The cells were incubated with the same treatment protocol for the flowcytometry experiment. The assay was triplicated in each treatment group. The absorbance (550 nm-690 nm) was quantified using a scanning multiwell spectrophotometer (Synergy2, BioTek). The cell-counting with flowcytometry described above was also performed in parallel with the same passage fibroblast samples. Both proliferation assays showed similar results (Figure A and B below).



A, Cultured rat fibroblast proliferation estimated by MTT assay after 1-hour and 24-hour treatment with vehicle-control, or 0.3 or 3-µmol/L Pyr3. Mean±SEM spectrophotometrical absorbance (550 nm-690 nm) (n=6, $P<0.05$ vs. CTL). B. Mean±SEM cell-count of rat fibroblast with flowcytometry after 1-hour and 24-hour treatment with vehicle-control, or 0.3 or 3-µmol/L Pyr3. (n=6, $P<0.05$ vs. CTL).

Western Blots

Total protein was extracted from freshly-isolated/cultured fibroblasts or atrial tissues, quantified, and processed as previously described. Cytoplasmic and nuclear protein fractions were extracted from fresh fibroblasts with ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). Protein-samples (20 µg/lane for total protein and 30 µg/lane for fractionated protein) were separated by 8% SDS-PAGE electrophoresis and then transferred to polyvinylidene-difluoride membranes. Membranes were blocked and incubated with mouse anti-α smooth-muscle actin (αSMA, 1/2000, Sigma), mouse anti-phospho p44/42-MAP-kinase (1/2000, Cell signaling), mouse anti-vimentin (1/1000, Santa Cruz), rabbit anti-TRPC3 (1/1000, Alomone), rabbit anti-TRPC1 (1/200, Alomone), mouse anti-TRPM7 (1/1000, Neuromab), mouse anti-NFATc3 (1/1000, Santa Cruz), rabbit anti-NFATc4 (1/1000,

Santa Cruz), mouse anti-HSP70 (1/1000, Stressgen), mouse anti-Lamin A/C (1/1000, Abcam), anti-GAPDH (1/10000, Fitzgerald), and rabbit anti-calsequestrin (1/2500, Dianova). Corresponding secondary antibodies conjugated to horseradish-peroxidase were used for detection. Staining was detected using chemiluminescence and quantified by video densitometry. All expression data are provided relative to GAPDH or calsequestrin staining for the same samples on the same gels.

Confocal Imaging

Cultured/freshly-isolated fibroblasts or left atrial tissue cryosections were fixed with 2%-paraformaldehyde, washed with PBS, and incubated with mouse anti-alpha SMA (1/400, Sigma), goat anti-vimentin (1/200, Santa Cruz), mouse anti-NFATc3 (1/200, Santa Cruz), rabbit anti-NFATc4 (1/200, Santa Cruz), followed by donkey anti-mouse IgG-Alexa Fluor 555 (1/600, Invitrogen), donkey anti-rabbit IgG-Alexa Fluor 488 (1/600, Invitrogen), donkey anti-goat IgG-Alexa Fluor 488 (1/600, Invitrogen), and TOPRO-3 iodide (1/500, Invitrogen). Apoptosis was assessed using TUNEL-assay with ApopTag plus fluorescein *in situ* apoptosis detection kits (Millipore). Fluorescent images were obtained via Zeiss LSM-710 or OLYMPUS Fluoview FV1000 inverted confocal microscope.

Fluorescent Ca^{2+}-Imaging

Primary cultured fibroblasts (1-day culture as described above) were loaded with Fluo-4 (10 µmol/L) in phenol-free M199-medium in the presence of pluronic acid (2.5 µg/ml) for 50 min at 37°C. Ca^{2+}-imaging was recorded with an Andor Revolution confocal system and a Xion camera (Andor Technologies) mounted on an upright Nikon FN-1 microscope with a 60× water-immersion objective (1.0 NA). Fluo-4 was excited with a 488-nm solid-state laser; emitted fluorescence was collected at 495 nm. Images (512×512 pixels) were acquired at 15 frames/sec for 5 min using iQ software (Andor

Technologies). Ca^{2+}-associated fluorescence was analyzed with custom-designed software (A. Bonev, University of Vermont). Regions of interest (ROIs) were determined by cell-outlines. F_O was determined by averaging fluorescence of ROIs from 10 consecutive baseline images.[5] At the end of experiments, ATP (100-µmol/L) was applied to verify cell-viability. One-day cultured fibroblasts were incubated in 2-mmol/L Ca^{2+}-containing Tyrode solution followed by Ca^{2+}-free solution to induce Ca^{2+}-store depletion and store-dependent Ca^{2+}-entry. Under OAG (25-µmol/L)- or angiotensin-II (100-nmol/L)-stimulation, cell-Ca^{2+} was recorded with confocal microscopy 5 minutes before and after re-administration of 2-mmol/L Ca^{2+} into the extracellular solution.

TRPC3-knockdown

Plasmid constructs

The TRPC3-specific shRNAmir over-expressing pGIPZ-based lentivirus vector plasmid was obtained from Open Biosystems (Oligo ID: V2LMM_11490).

The scrambled shRNAmir over-expressing plasmid was generated as follows. The empty pGIPZ lentivirus vector plasmid carrying the mRNA-context sequence but no shRNA was obtained from Open Biosystems. The EcoRI site of pGIPZ located at position 5394 was removed by partial EcoRI digestion and Klenow fill-in, resulting in pGIPZΔEcoRI. This modification allowed the direct cloning of the scrambled shRNAmir construct in pGIPZ between XhoI, 2654 and EcoRI, 2678 sites. In the design of the scrambled shRNAmir and during the rest of the cloning procedure, we followed the methods by Paddison et al.[6] Briefly, a 97bp long synthetic oligonucleotide

(5`TGCTGTTGACAGTGAGCG*CCGATATCAGCAGATAATGAAA*TAGTGAAGCCACAGATGTA

TTTCATTATCTGCTGATATCGTTGCCTACTGCCTCGGA 3`, *passanger strand*, loop, guide strand)

was PCR amplified by 5`CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG sense and

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

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

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

Lentivirus production

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

***miR-26a*-Overexpression/Knockdown**

For the *miR-26a* overexpression, the sense (5` UUCAAGUAAUCCAGGAUAGGCU 3`) and antisense (5` CCUAUUCUUGGUUACUUGCACG 3`) oligoribonucleotides were synthesized by Invitrogen. The both strands were annealed by mixing the same volume of each oligoribonucleotides dissolved in annealing buffer (IDT, Coralville, IA) at a concentration of 200 μmol/L and then incubated at 95°C for 10 min, 70°C for 10 min and then 50°C for 10 min. A scrambled RNA was used as negative control (sense: 5` UCAUAAAGCUGAUAACCUCUAGAU 3`, antisense: 5` CUAGAGGUUAU CAGCUUUAUGAAU 3`).

For the *miR-26a* knockdown, the antisense anti-miRNA oligonucleotides (AMO26a: 5` AGCCTATCCTGGATTACTTGAA 3`) was synthesized by Exiqon (Exiqon, Woburn, MA). Five oligonucleotides on 5`-end of the antisense molecules and four oligonucleotides on 3`-end were locked by a methylene bridge connecting the 2'-O atom and 4' -C atom (Locked Nucleic Acid, LNA). A scrambled oligonucleotides with the same methylene bridge were used as negative control (5` ACTCAGAAGGACAAGTAGAGTCT 3`).

Dog left-atrial fibroblasts in primary culture were transfected with miR-26a (final concentration of 100 nmol/L) and/or AMO26a (final concentration of 10 nmol/L), and negative control miRNAs or AMOs with lipofectamine 2000 (Invitrogen).[7] After 48-hour transfection, cells were collected for total RNA/protein purification or proliferation analysis with a FACScan. The efficacy of miR-26a overexpression/knockdown by the miR-26a probes was confirmed by qPCR before experiments (Supplemental Figure 12C).

Dual Luciferase Reporter Assay

A fragment (5` TGACTATAGCACAAATGTGGGCAATAATATTTCTAAGTATAAAATACTT GAAATGGTGTAAATTTTTAGTATTAACTACCTTTATCATGTGAATCTTTAA 3` miR-26a target site) containing miR-26a target gene of TRPC3 was synthesized by Invitrogen and was used as a template for PCR amplification with 5` GACTAGTTGACTATAGCA CAAATGT 3` and 5` CCCAAGCTTTTAAAGATTCACATGATA 3`as forward and reverse primers, respectively. The PCR product was ligated into HindIII and SpeI sites (3` UTR region of luciferase gene) in the pMIR-REPORT luciferase miRNA expression reporter vector (CMV promoter-driven Firefly luciferase expression vector, Applied Biosystems) and was subcloned.

For luciferase assay, HEK293 cells were simultaneously transfected with 50 ng of pMIR-REPORT, 0.5 ng pRL-TK (TK promoter-driven Renilla luciferase expression vector, Promega, Madison, WI) and miR-26a (final concentration of 10 nmol/L) and/or AMO26a (final concentration of 3 nmol/L) with lipofectamine 2000 (Invitrogen). After 48-hour transfection, luciferase activities were measured with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany).[7] The pMIR-REPORT firefly luciferase signals were normalized to the pRL-TK renilla luciferase signals as an internal control.

Statistical Analyses

Data are presented as mean±SEM. Two-way ANOVA with multiple-group comparisons (Bonferroni-corrected t-test) was data with two or more main-effect factors like flow-cytometry with varying culture-times (treatment and time as main-effect factors) and ERP (basic cycle length and dog-group main factors). One-way ANOVA was applied for single main-effect factor experiments like Western blots, qPCR, and luciferase assay. Repeated-measure analyses were used when the same set of

subjects/materials were exposed to multiple interventions. Students' *t*-tests were used for comparisons involving only two groups. For multiple comparisons with Bonferroni correction, adjusted *P*-values were calculated as multiplying original *P* values for each pairwise comparison by the number of comparisons (*N*) performed; values shown are adjusted values ($N \times P$). Two-tailed $P<0.05$ was considered to be statistically significant. All analyses were performed with SPSS 16.0.

Specific Analyses for Individual Figures and Tables:

Two-way ANOVA with multiple comparison (Bonferroni-adjusted t-tests) was used for data with two or more factors as main effects and repeated measures (Figures 1D, 1E and 1F, Figures 2B-2E, Figure 3B, Figure 4B, Figure 5A, 5B, 5D, 5E, Figure 8B, 8D and 8E, Supplemental Figure 4B, Supplemental Figure 5C and 5D, Supplemental Figure 7B and 7C, Supplemental Fig 8C and 8D).

One-way ANOVA was applied for data with a single main-effects factor as obtained in studies of heart rate and echocardiographic data over time, with one main factor and 3 levels (groups) or more (Supplemental Fig 11A, 11C-11E). Repeated-measure analyses were used when the same set of subjects/materials were exposed to multiple interventions (Figure 2A, Figure 6B-6G, Supplemental Figure 12C).

Paired t-tests were used for Figures 1H, Figure 5F, Figures 7D and 7E, and Supplemental Figures 6B, 7D, 8A and 8B.

Non-paired t-tests were used for Figures 3C, 3E-G, Figures 4C-E, Figure 5C, 5G, Figure 7B, 7C, Figures 8A, 8C, and 8F, and Figures 9A-I as well as Supplemental Table 1.

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Supplemental Table 1. Hemodynamic Data

	Control (n=11)	AF (n=12)
Body weight, kg	26.8±0.3	28.2±0.2
Systolic BP, mmHg	127±2	120±2
Diastolic BP, mmHg	73±2	76±2
RAP, mmHg	1.3±0.1	2.5±0.1*
LAP, mmHg	2.6±0.1	6.5±0.3*
RVP, mmHg	22±0.4	23±0.5
RVEDP ,mmHg	1.5±0.1	4.2±0.3*
LVP, mmHg	116±1.4	101±1.9
LVEDP, mmHg	3.9±0.2	8.0±0.5*

*$P<0.05$ vs control.

BP indicates blood pressure; RAP and LAP, RA and LA mean pressure; RVP and LVP, RV and LV mean pressure; RVEDP and LVEDP, RV and LV end diastolic pressure.

Supplemental Figure Legends

Supplemental Figure 1. A, mRNA expression of TRP channels measured by Taqman Low-density array. B, mRNA expression of TRPC3 channel on enlarged scale.

Supplemental Figure 2. A, Representative immunofluorescent images of freshly-isolated rat cardiac fibroblasts. Red indicates α-smooth muscle actin (αSMA); green, vimentin; blue, TOPRO-3 (nuclear staining). B, Representative immunofluorescent images of passage-cultured myofibroblasts. C, Representative I_{NSC}-recordings with or without gadolinium (Gd^{3+}, 100-μmol/L) in freshly-isolated rat cardiac fibroblasts. Voltage-clamp protocol is shown in the inset. D and E, Representative Gd^{3+}- and Pyr3-sensitive I_{NSC} in passage-cultured myofibroblasts. F, Mean±SEM Gd^{3+}- and Pyr3-sensitive I_{NSC} density (n=10 cells in Gd^{3+} and 8 cells in Pyr3).

Supplemental Figure 3. Protocol for in vivo TRPC3-blocker (pyrazole-3) treatment study in AF dogs.

Supplemental Figure 4. A, Recordings of OAG-induced intracellular Ca^{2+}-response in presence or absence of Pyr3. B, Mean±SEM OAG-induced Ca^{2+} fluorescence (F/F_0), normalized to baseline intensity under 0-mmol/L and 2-mmol/L Ca^{2+} (n=14 cells in each condition, **P*<0.05 vs. 0-mmol/L Ca^{2+}, #*P*<0.05 vs. 2-mmol/L Ca^{2+} in OAG-only).

Supplemental Figure 5. A and B, Representative DNA-content histograms and Dean-Jett-Fox model fitting of rat cultured fibroblasts with or without 24-hour treatment of Gd^{3+} (100-μmol/L) or Pyr3 (3-μmol/L). C, Mean±SEM cell-count of rat fibroblasts after 1-hour and 24-hour culture with vehicle-control, or 10 or 100-μmol/L Gd^{3+} (n=8/group, **P*<0.05 vs. CTL, #*P*<0.05 vs. 1-h treatment). D, Mean±SEM percentage of cells in G2/M-phase after Gd^{3+} treatment (n=8/group, **P*<0.05 vs. CTL, #*P*<0.05 vs. 1-h treatment).

Supplemental Figure 6. A, Representative images of TUNEL staining in cardiac rat fibroblasts with or without Pyr3 treatment (3-µmol/L). Red indicates TOPRO-3 (nuclear staining); yellow, TUNEL-positive cells. B, Mean±SEM percentage of TUNEL-positive cells. (n=5/group, **P*<0.05 vs. CTL).

Supplemental Figure 7. A, Representative immunofluorescent images of rat cardiac fibroblasts cultured with or without Pyr3 (3-µmol/L). Red indicates α-smooth muscle actin (αSMA); blue, TOPRO-3 (nuclear staining). B, Mean±SEM cell-count of culture-passaged fibroblasts after 1-hour and 24-hour treatment with 3-µmol/L Pyr3 or vehicle DMSO (n=6/group). C, Mean±SEM percentage of culture-passaged fibroblast cells in G2/M-phase after Pyr3 treatment or vehicle DMSO (n=6/group). D, Representative immunoblots (left) and mean±SEM αSMA-expression in culture-passaged fibroblasts (right, normalized to GAPDH) (n=6).

Supplemental Figure 8. A, Mean±SEM TRPC3 and TRPC6 mRNA-expression in scrambled (Scr)-shRNA- and TRPC3-shRNA-infected dog atrial fibroblasts (n=6/group, **P*<0.05 vs. scrambled shRNA). B, Top: Immunoblots for TRPC3 subunits and GAPDH. Bottom: Mean±SEM TRPC3/GAPDH protein-expression (n=6, **P*<0.05 vs. scramble shRNA). C, Mean±SEM fold-change over time in the number of fibroblasts, following infection with shRNA or scrambled-probe virus (n=6/group, **P*<0.05 vs. scrambled shRNA). D, Mean±SEM percentage of virus-infected fibroblast cells in G2/M-phase (n=6/group, **P*<0.05 vs. scrambled-shRNA).

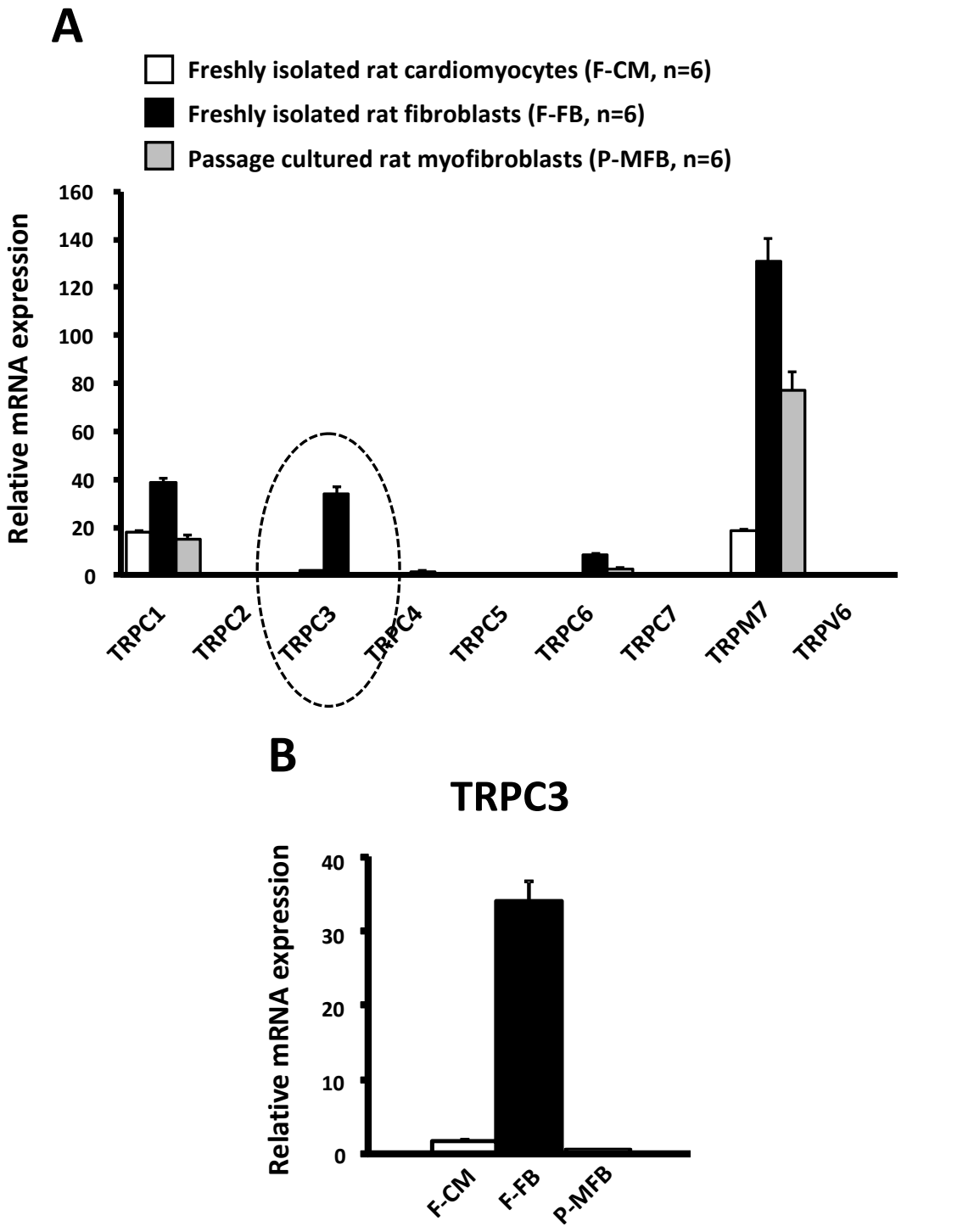
Supplemental Figure 9. A, through C, Representative immunoblots (top) and mean±SEM (bottom) TRPC3 subunit protein-expression (normalized to calsequestrin, CSQ) in right-atrial samples from patients with AF (n=8) and in sinus rhythm (n=10), AF model goats (n=8) and normal goats (n=5) and congestive heart failure dogs with AF substrates (n=8) and normal dogs (n=8) (**P*<0.05 vs. control). D through F, TRPC1 subunit expression. G through I, TRPM7 subunit expression.

Supplemental Figure 10. A-E, Representative surface ECGs (left) and intracardiac electrograms (right) in an AF dog on Day 0 through Day 7.

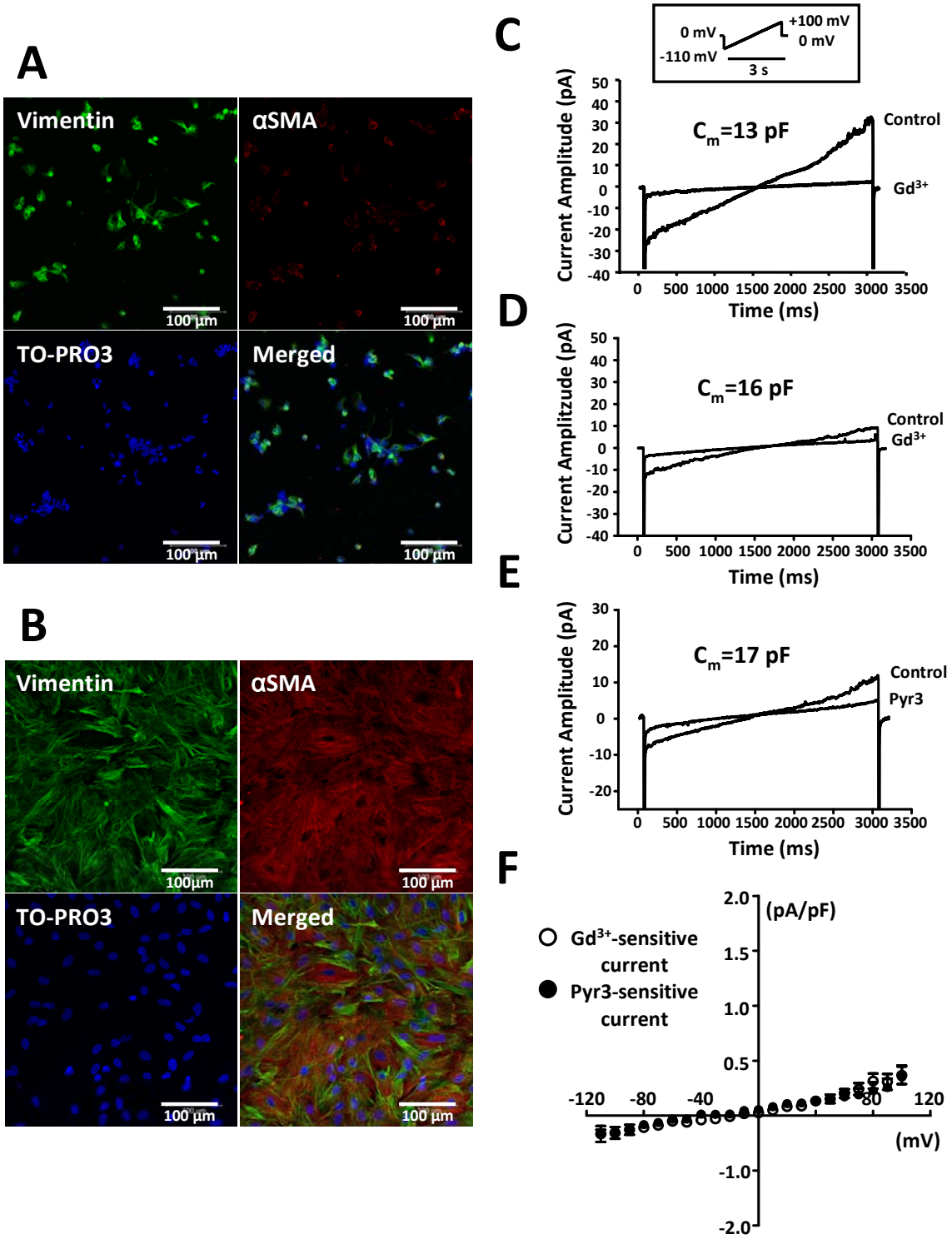
Supplemental Figure 11. A, Mean±SEM heart rate over time during atrial tachypacing, ATP (n=7 CTL and 12 AF, **P*<0.05 vs. CTL). B, Representative apical 4 chamber views of echocardiography on Day 0 (baseline, before pacing, left) and Day 7 (right) in an AF dog. C, Mean±SEM atrial diastolic area (n=10 CTL and 11 AF, *P*<0.05 vs. CTL). D, atrial fractional area change. E, ventricular diastolic volume.

Supplemental Figure 12. A, miR-26 and its complementary sequence on TRPC3 3'-UTR region. Sequences highlighted in yellow represent the seed region of miR-26a. B, Antisense anti-miR-26a oligonucleotide (AMO26a) sequence and its complementarity to miR-26. Sequences highlighted in yellow represent the seed region of miR-26. Bold characters in the AMO26a sequence indicate locked nucleic acids (LNAs). C, Results of miR-26a overexpression/knockdown by miR-26a duplex/AMO26a in dog left-atrial fibroblasts. Lipo; lipofectamine transfection without the miR-26a probes. (n=4, *P*<0.05 vs. Lipo). D, Multiple putative NFAT binding motifs in the promoter regions of the host gene for miR-26a and miR-26b in human and canine genomes. Ctdsp: Carboxy-Terminal Domain RNA polymerase II polypeptide A Small Phosphatase. The numbers before the sequences represent the relative positions of predicted NFAT binding motifs to the transcription start site (TSS) of the host gene. The numbers in the brackets stand for the scores given by Genomatix (http://www.genomatix.de) for the similarity between the putative NFAT cis-acting elements in the promoter regions of the host genes for miR-26a/b and the perfect NFAT binding sequence; Two scores are given as core sequence similarity and overall sequence similarity. Only those with overall matrix similarity >0.95 are shown. 5000 bp upstream to the predicted TSS for miR-26a/b host genes in human and canine was analyzed.

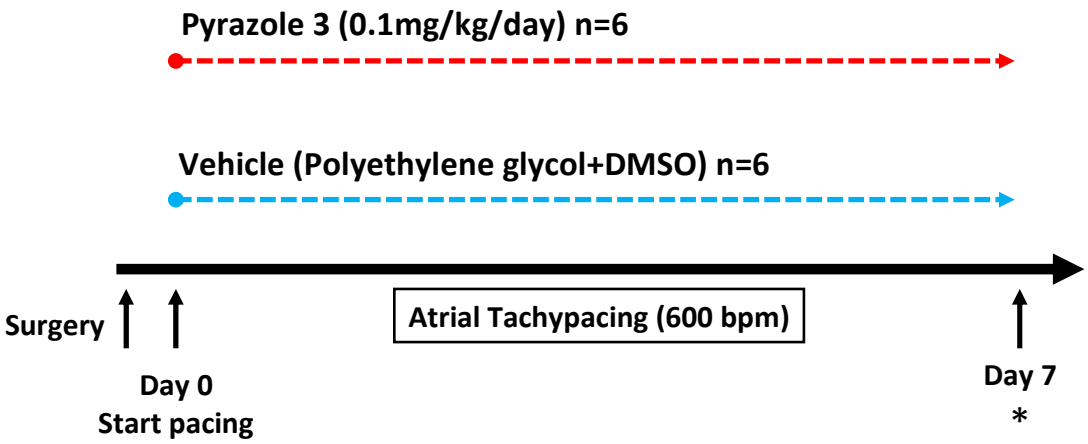
Supplemental Figure 1



Supplemental Figure 2



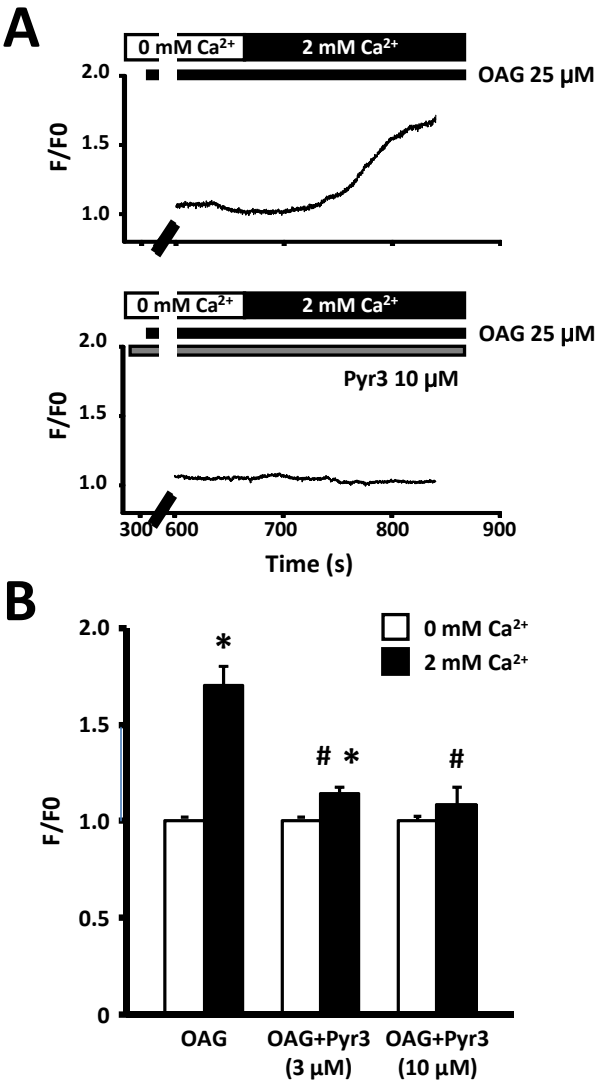
Supplemental Figure 3



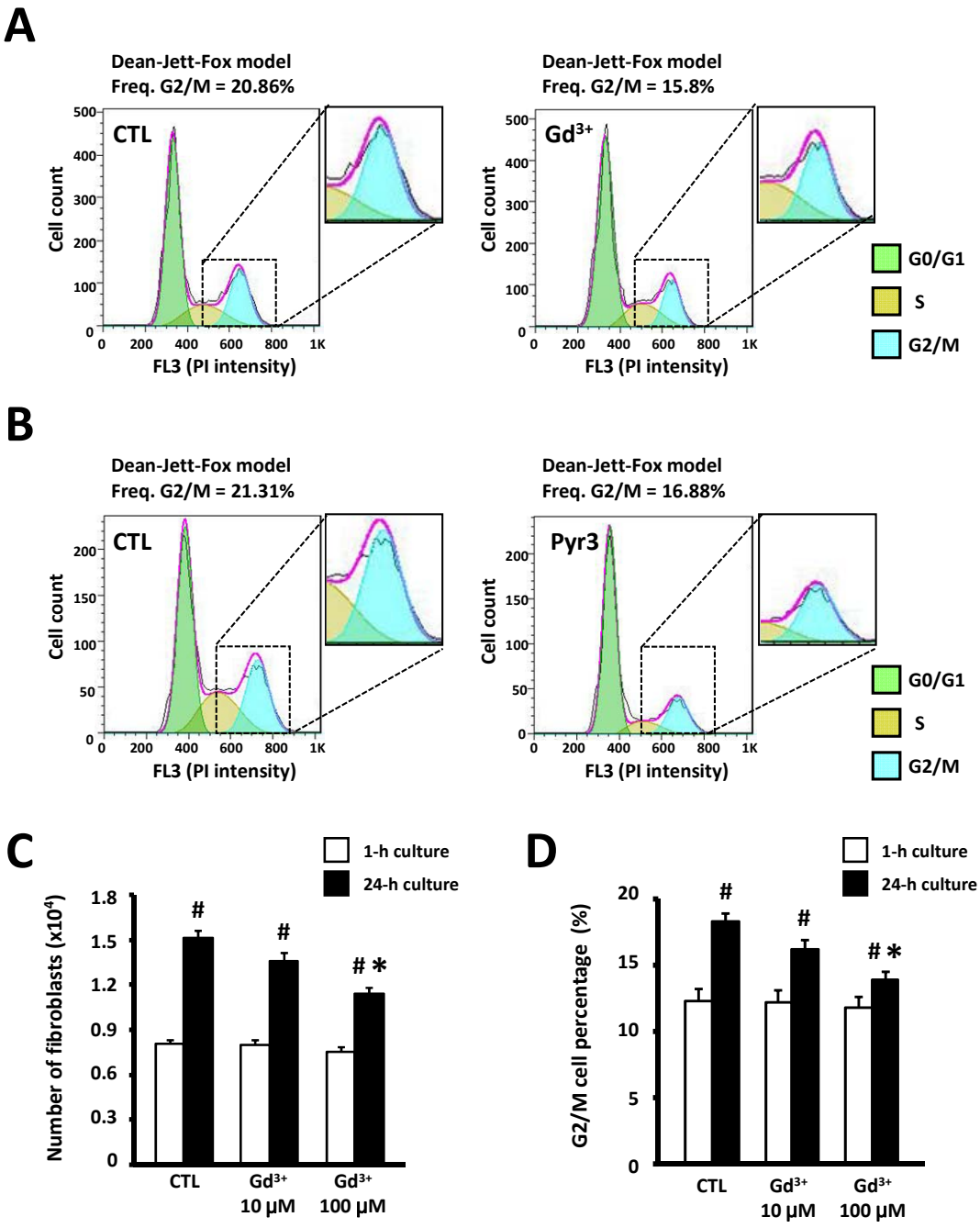
Drugs were continuously administrated with Alzet Osmotic Pump.

<u>*Terminal Experiments</u>
Open chest EP study
Vimentin Western Blotting in LA tissue sample
qPCR for collagen gene in fresh fibroblasts
Proliferation assay in cultured fibroblasts

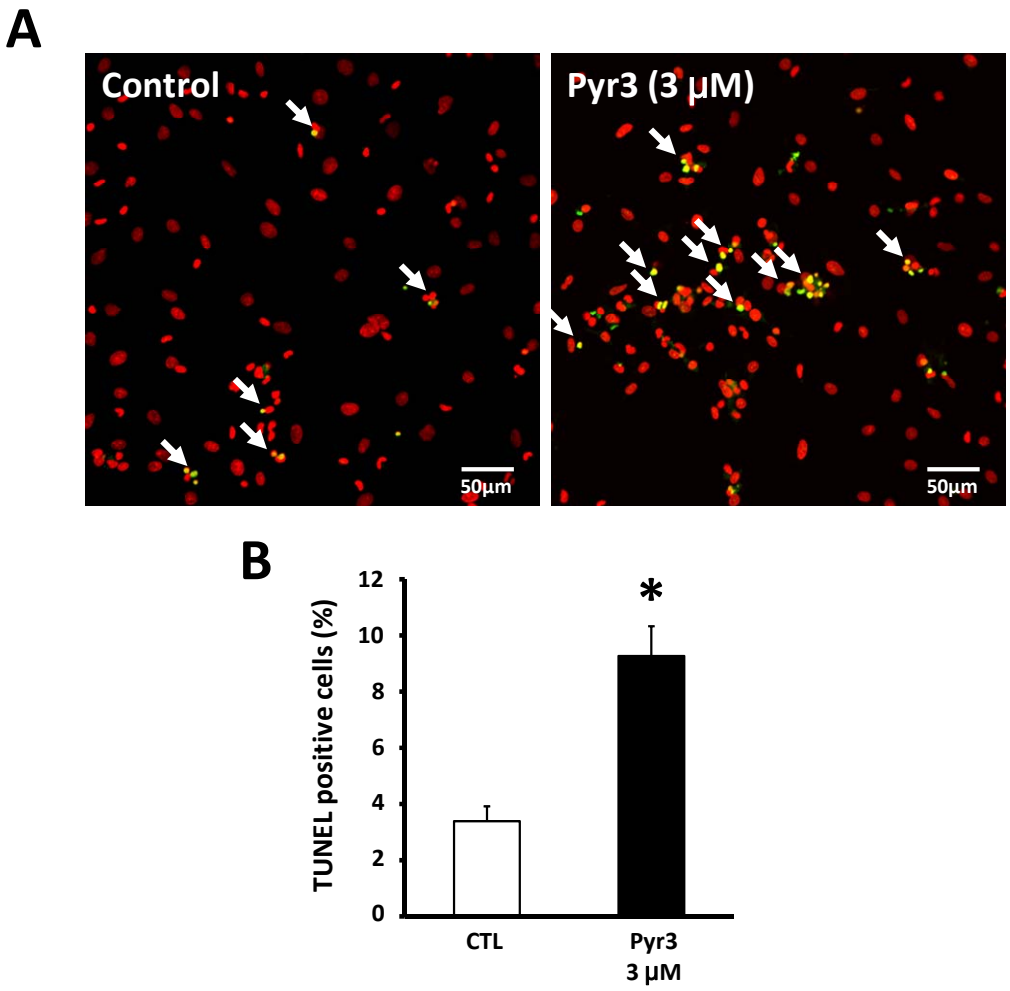
Supplemental Figure 4



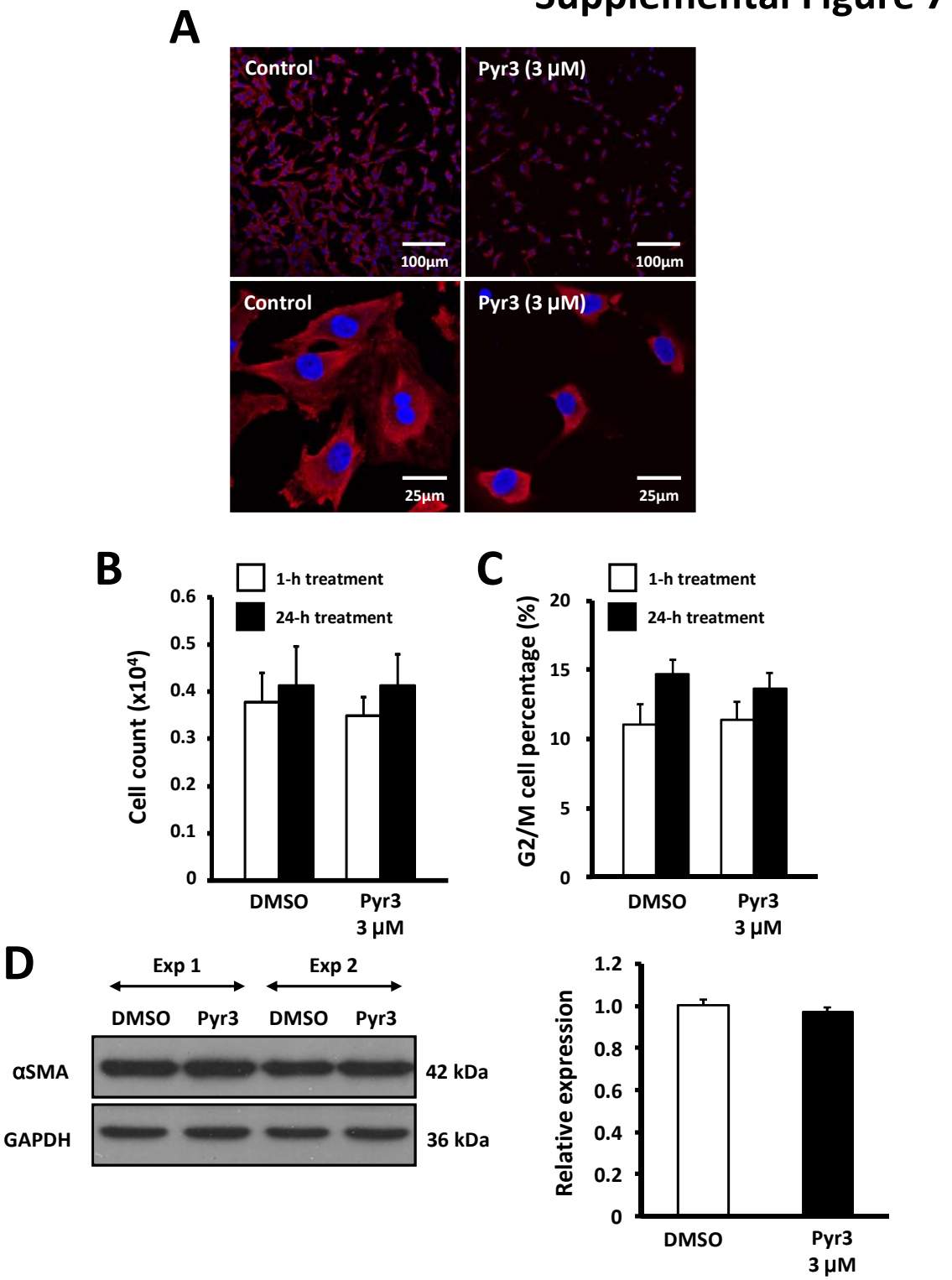
Supplemental Figure 5



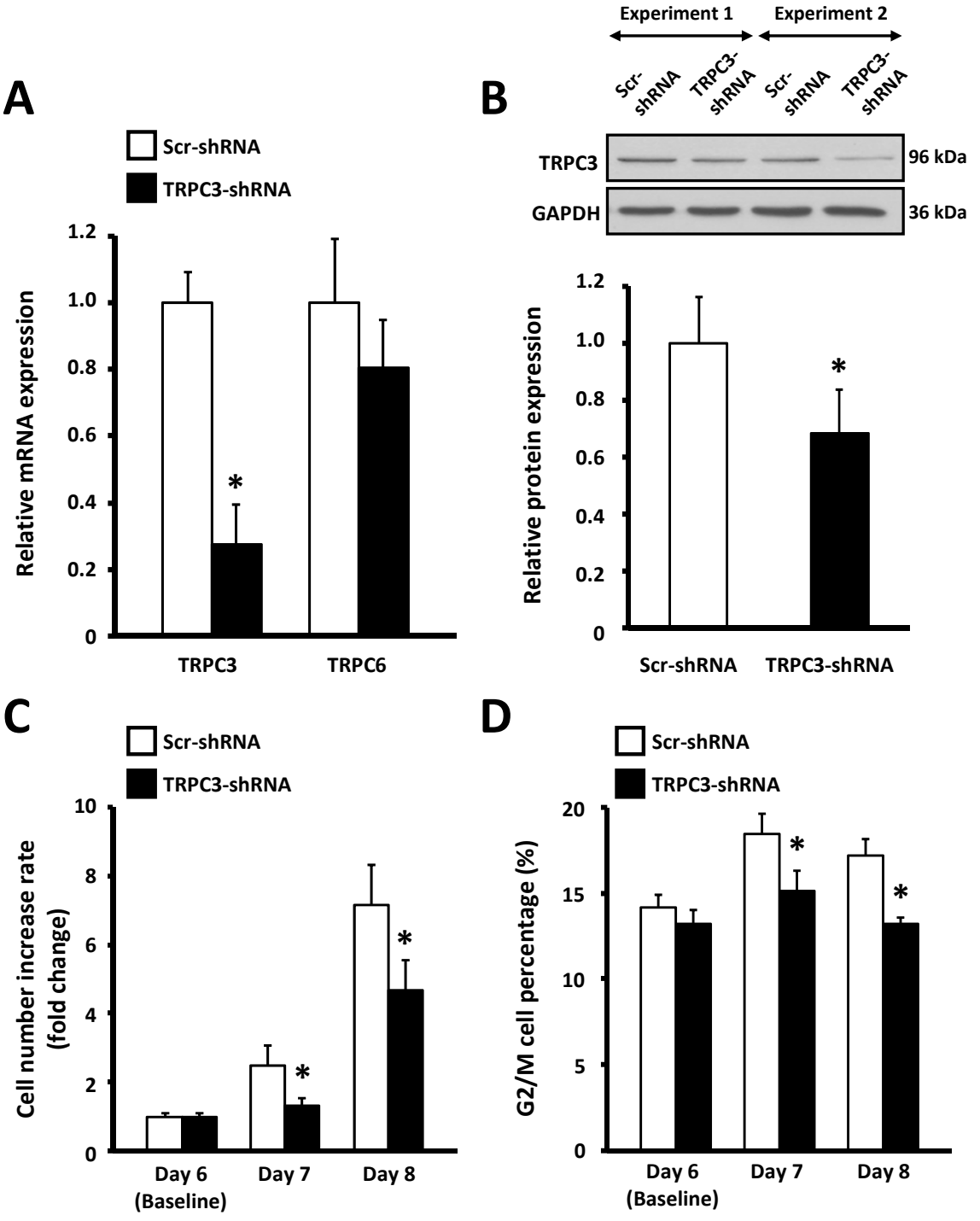
Supplemental Figure 6



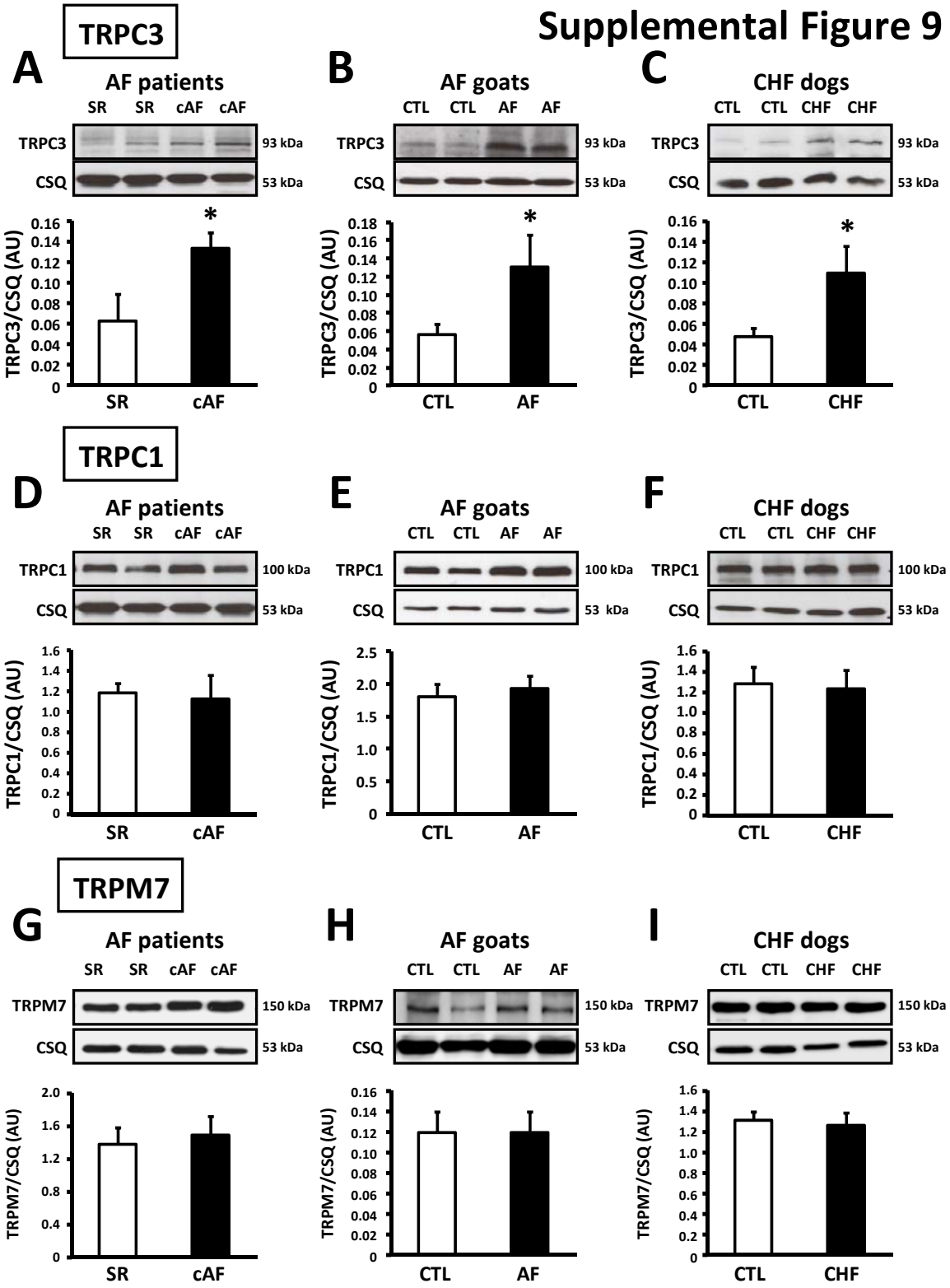
Supplemental Figure 7



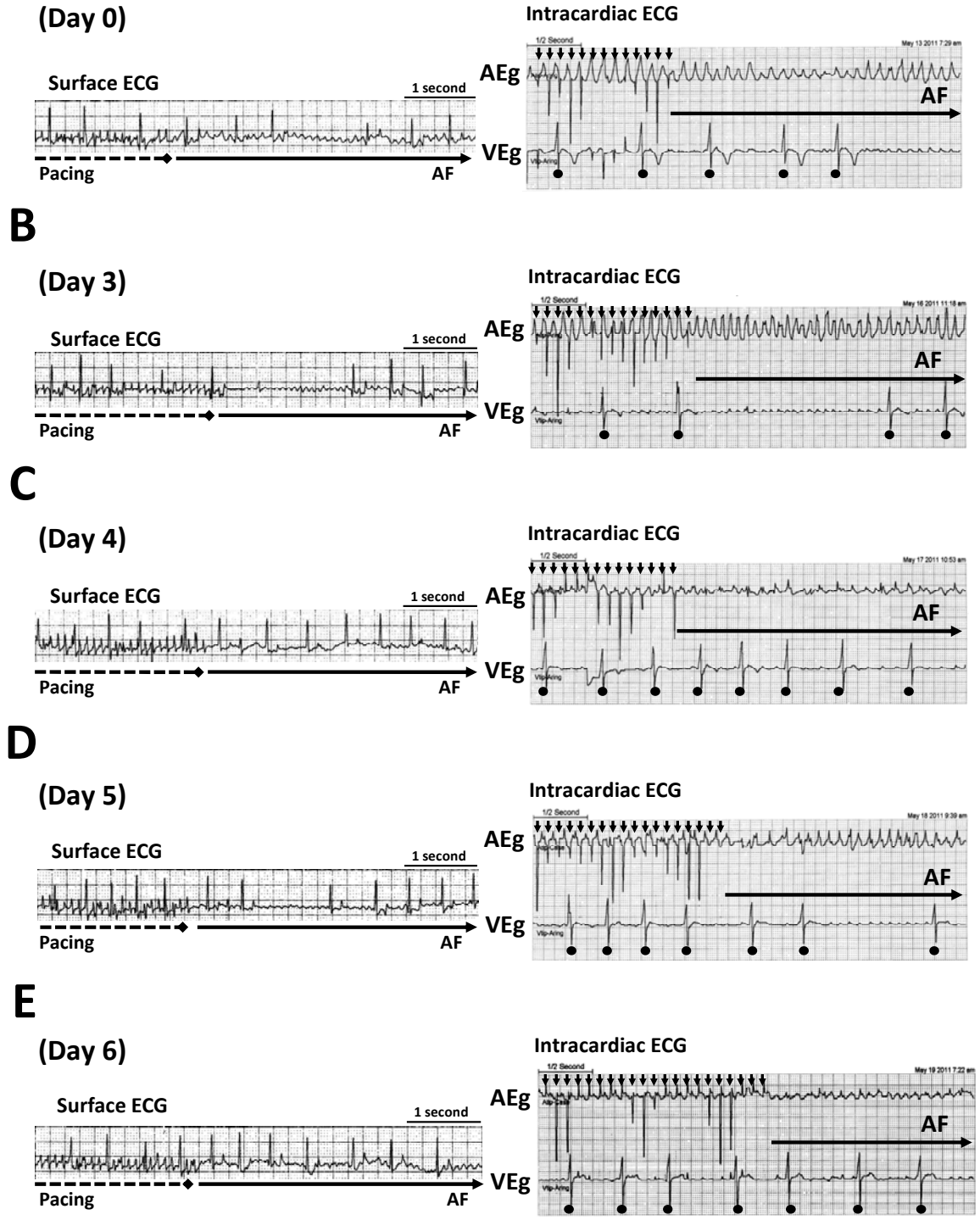
Supplemental Figure 8



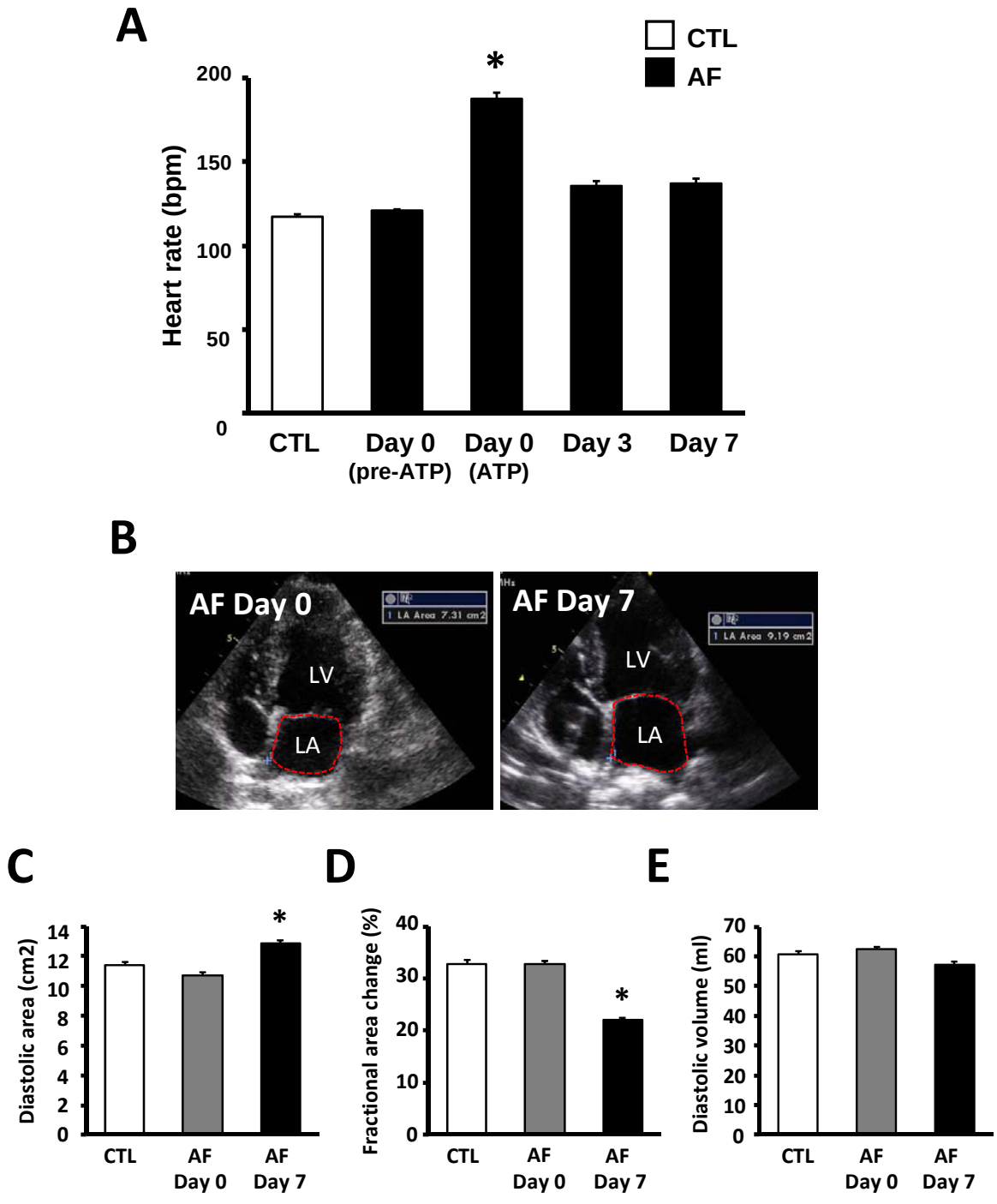
Supplemental Figure 9



Supplemental Figure 10



Supplemental Figure 11



Supplemental Figure 12

A

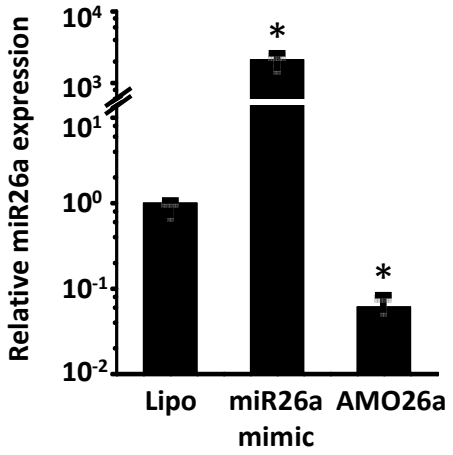
```
3′-UCGGAUAGGACCUAAUGAACUU-5′  miR-26a
     |: | : || | ||||||||
5′-UUCUAAGUAUGAAAUACUUGAA-3′  TRPC3 3'UTR (Human)

3′-UCGGAUAGGACCUAAUGAACUU-5′  miR-26a
     |: | : |  | ||||||||
5′-UUCUAAGUAUAAAAUACUUGAA-3′  TRPC3 3'UTR (Canine)
```

B

```
5′-UUCAAGUAAUCCAGGAUAGGCU-3′  miR-26a(Human)
5′-UUCAAGUAAUCCAGGAUAGGCU-3′  miR-26a(Canine)
   ||||||||||||||||||||||
3′-AAGTTCATTAGGTCCTATCCGA-5′  AMO26a
   ||||||||||:||||||||||:|
5′-UUCAAGUAAUUCAGGAUAGGUU-3′  miR-26b(Canine)
5′-UUCAAGUAAUUCAGGAUAGGU-3′   miR-26b(Human)
```

C



D

Host gene (miRNA)	Human	Canine
Ctdspl (miR-26a-1)	4430-GTGATGGAAACATTTGGAG (1/0.97) 4040-ATGGAGGAAATTAGAGATG (1/1) 3992-CAGGTGGAAAGGCACTCCA (1/0.95) **3340-CATGAGGAAAAAATATATC** (1/0.96)	2922-GCGGAGGAAAAAGTAGCTG (1/0.99) 1590-GCTGAGGAAATAAACTAGG (1/1) 1479-CCTCAGGAAACAGTGGTCA (1/0.95)
Ctdsp2 (miR-26a-2)	4480-TTGGAGGAAAGGAGAATGA (1/0.97) **3618-CCAGAGGAAAGGGGATACA** (1/0.97) 3385-CTATAGGAAAGAGGCCCAC (1/0.96)	2529-GTTGGAAACAGGGAAGCCT (1/0.99)
Ctdsp1 (miR-26b)	1134-GGAGAGGAAAGCGCGCCAC (1/0.97) **373-AACGAGGAAAAAGACCAAC** (1/0.99)	209-GGGGAGGAAACTCCATGTT (1/0.97) 190-GCGGAGGAAACTTTGTTAC (1/0.97)