Supplemental Material

Effects of atrial fibrillation on the human ventricle

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Supplementary Materials and Methods

RNA isolation: Pellets of iPSCs, and iPSC-CMs were snap frozen and stored at -80°C. The RNA of iPSCs was isolated with the SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. In short, the pellets were lyzed with 400-600 µl RNA lysis buffer supplemented with β-ME (200 µL/10 mL). Equal volumes of 95%-ethanol were added to the lysate and it was then transferred to a spin basket tube. The samples were washed twice and the RNA was eluted with 100 µL of nuclease free water. For iPSC CMs the ReliaPrep RNA Tissue Miniprep System (Promega) was used as stated by the manufacturer's protocol.

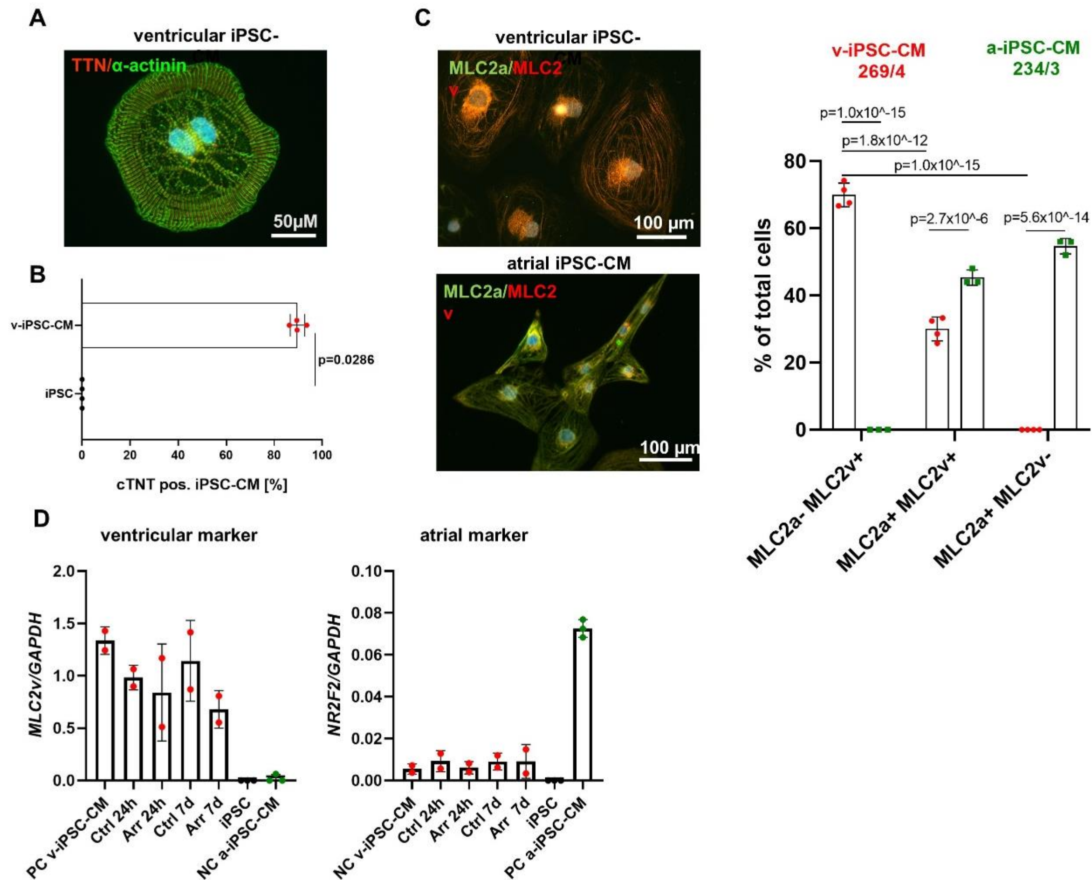
After RNA isolation, the RNA was cleaned and concentrated using the RNA Clean & Concentrator -5 System (Zymo Research) according to the manufacturer's protocol. In short, the RNA was incubated with DNAse I and DNA digestion buffer for 15 minutes at room temperature (RT). Two volumes RNA binding buffer were added to each sample. Equal volumes of 95%-ethanol were added before transferring the samples to a spin basket tube. RNA prep buffer was added and the samples were washed once using RNA wash buffer. The RNA was eluted with 15 µl of nuclease free water. RNA concentration was determined with a spectrophotometer at 260/280 nm and the samples were stored at -80°C.

Gene expression analysis: The iScript cDNA Synthesis Kit (Bio-Rad) was used according to the manufacturer's protocol to receive cDNA from our isolated RNA. Gene expression of cardiac markers was tested with qualitative real-time PCR (qRT-PCR). DNA standards with concentrations ranging between 1 ng/µL to 0.125 fg/µL served as a reference. For our assays

we used the CFX Connect Real-Time System (Bio-Rad). The PCR reaction went through 40 cycles of 10 sec at 95°C followed by 20 sec at 60°C and 30 sec of 72°C. Fluorescence was measured after each cycle. Specificity of the products was ensured through a melt curve analysis at the end of the run. Threshold cycles calculations and quantification of DNA was done by the system's software CFX Maestro 1.1. Detailed primer sequences are listed in Supplementary Table 1.

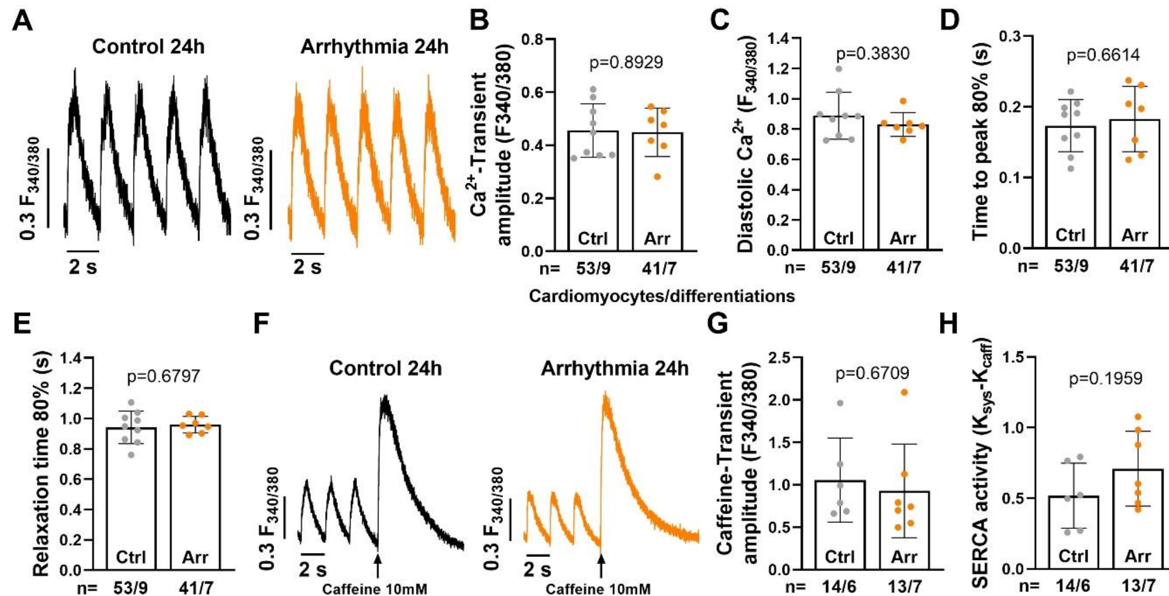
Immunocytochemistry staining: Occurrence and location of cellular proteins was shown with immunocytochemistry staining as described earlier by our group. In short, CMs were cultured on glass cover slips, fixed with 4% paraformaldehyde (PFA, Sigma) in PBS at room temperature for 20 min and blocked with 1% BSA/PBS overnight at 4°C. When staining intracellular proteins, cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. All samples were incubated with the primary antibody overnight at 4°C in a humidified chamber. For the secondary antibody the incubation time was 1 hour at 37°C. The nuclei were counterstained with 4.6-diamino-2-phenylindole (DAPI, 0.2 ng/mL, Sigma) for 10 min at room temperature in the dark. Finally, the cover slips were mounted onto microscopy slides with Vectashield Mounting Medium (Liniaris Biologische Produkte GmbH) and sealed with nail polish. Images were captured with a Zeiss Axio Observer.Z1 microscope and a Zeiss AxioCam MRm 1.4MP camera. A detailed list of the used antibodies is attached (Supplementary Table 2).

For the analysis of ratio of MLC2a+/MLC2v+ cardiomyocytes, a double staining using antibodies against both proteins was performed. 50-60 cells per differentiation were randomly chosen and analyzed regarding their expression of MLC2a and MLC2v individually. Specificity of the antibodies for immunofluorescence staining was tested by performing the same staining without secondary antibodies for MLC2v, MLC2a and a-actinin. Moreover, we performed flow cytometry analysis using antibodies against the cardiac marker cTNT. Here, we tested specificity of the antibody by using isotype controls. Statistics were performed with GraphPad Prism 8.



Supplemental Figure 1: Quality control (structural analysis and quantitative analysis of gene and protein expression) of generated ventricular iPSC-CM. **(A)** Immunofluorescence staining of iPSC-derived ventricular cardiomyocytes (v-iPSC-CM) for titin (TTN), and α-actinin; nuclei were stained with DAPI. Scale bar= 50 µM. **(B)** Quantitative Flow cytometry analysis of v-iPSC-CM for cardiac troponin T (cTNT). IPSCs served as negative control. n= 4 independent differentiation experiments from 3 iPSC lines. Data are analyzed by the Mann-Whitney Test. **(C)** Structural characterization via immunofluorescence staining of v-iPSC-CM and atrial differentiated iPSC-CM (a-iPSC-CM) for MLC2v, MLC2a; nuclei were stained with DAPI. Right: Quantification of MLC2v+ and MLC2a+ cells in immunofluorescently stained cultures (n = 4 ventricular (269 cells) and n = 3 atrial (234 cells) independent differentiation experiments from 3 iPSC lines, day 90). Data are tested by the 2-way ANOVA test. Scale bar= 100 µM. **(D)** QPCR analysis for subtype markers *MLC2V* (ventricular) and *NR2F2* (atrial) at day 60-90 in iPSC, unstimulated ventricular and atrial iPSC-CM (v-iPSC-CM, a-iPSC-CM, 2-3 independent

differentiations) and in ventricular iPSC-CM after arrhythmic (Arr) or rhythmic stimulation (Ctrl) for 24 h or 7 days. Data are presented as mean ± SD.



Supplemental Figure 2: Human induced pluripotent stem cell cardiomyocytes (iPSC-CM) after 24 h atrial fibrillation (AF)-simulation (arrhythmic pacing: Arr; 60 bpm, 40% beat-to-beat-variability) or rhythmic pacing (control (Ctrl); 60 bpm). **(A)** Representative recordings of stimulated Ca^{2+} transients (epifluorescence microscopy, Fura-2) and **(B)** mean values ± SD for Ca^{2+} transient amplitude, **(C)** diastolic Ca^{2+} levels, **(D)** time to peak 80%, **(E)** relaxation time 80% of human iPSC-CM upon chronic AF-simulation (n=41 cardiomyocytes/7 differentiations/4 donors) or rhythmic pacing (n=53/9/4). **(F)** Original recordings of caffeine-induced Ca^{2+} transients (10 mM caffeine, epifluorescence microscopy, Fura-2) and **(G)** mean values ± SD for caffeine-transient amplitude indicating the sarcoplasmic reticulum Ca^{2+} load of iPSC-CM after chronic AF-simulation (n=13/7/4) or rhythmic pacing (n=14/6/4). P-values were calculated using unpaired t-test.

Supplementary Table 1: Used primers for qPCR

Gene	5´-3´Sequence	bp	Anneal. Temp/Cycles
MLC2v	GGCGAGTGAACGTGAAAAAT/ CAGCATTTCCCGAACGTAAT	200	60°C/40
NR2F2	CCGACCGGGTGGTCGCCTTTATGGA/ CGGCTGGTTGGGGTACTGGCTCCTA	223	60°C/40
GAPDH	GTCTCCTCTGACTTCAACAGCG/ ACCACCCTGTTGCTGTAGCCAA	110	60°C/40

Supplementary Table 2: Used antibodies for immunofluorescence

	Primary antibodies			
Protein	**Manufacturer**	**Dilution**	**Host**	**Method**
αActinin (monoclonal, IgG1)	Sigma (#A7811)	1:500	mouse	IF
TitinM8/M9 (polyclonal, IgG)	Myomedix (order form)	1:750	rabbit	IF
MLC2v (polyclonal, IgG)	Proteintech (#10906-1-AP)	1:200	rabbit	IF
MLC2a (monoclonal, IgG2B)	Synaptic Systems (#311 011)	1:200	mouse	IF

cTNT (monoclonal, IgG1)	Thermo Fisher Scientific (#MS-295-PABX)	1:500	mouse	FLOW
	Secondary antibodies			
Protein	**Manufacturer**	**Dilution**	**Host**	**Method**
Donkey-anti-mouse IgG (H+L) AlexaFluor488	LifeTechnologies (#A21202)	1:1000	donkey	IF, FLOW
Goat-anti-mouse IgG/IgM (H+L) AlexaFluor488	LifeTechnologies (#A10680)	1:500	goat	IF
Donkey-anti-rabbit IgG (H+L) AlexaFluor555	LifeTechnologies (#A31572)	1:750	donkey	IF