Decreased intercellular coupling improves the function of cardiac pacemakers derived from mouse embryonic stem cells

John P. Fahrenbach[1], Xun Ai, and Kathrin Banach[2]

Supplementary Material and Figures

Intracellular Ca measurements

To visualize excitation-dependent changes of the intracellular Ca concentration ($[Ca]_i$), HL-1 monolayers were incubated (20 min) at room temperature with 5 µM fluo-4 acetoxymethyl ester (fluo-4/AM; Invitrogen, Eugene, OR, USA). After washout, twenty minutes were allowed for de-esterification of the dye. $[Ca]_i$ measurements were performed as previously described [1, 2]. Whole-cell $[Ca^{2+}]_i$ transients were obtained from confocal linescan images through HL-1 cell monolayers by averaging the signal of an individual cell. $[Ca^{2+}]_i$ transients are presented as background-subtracted normalized fluorescence (F/F_0).

Electrophysiological Recordings

HL-1 cells or ESdCs were cultured on 25mm glass coverslips. Patch electrodes with 3-5 MΩ tip resistances were pulled from borosilicate glass (WPI) and filled with a solution containing (mM): potassium aspartate 120; KCl 8; Na_2ATP 5; $MgCl_2$ 1; Hepes 10, pH adjusted to 7.2 with KOH. Cultures were perfused at room temperature (22-23ºC) with a physiological salt solution containing (mM): NaCl 140; KCl 5.4; CaCl2 1.8; MgCl2 1.2; glucose 10; Hepes 5, pH adjusted to 7.35 with NaOH. Upon obtaining the whole-cell configuration with the patch clamp technique, spontaneous action potentials (AP) were recorded with an Axon MultiClamp 700A in the current clamp mode. APs were sampled at 10 kHz and analyzed off-line with custom programs written for

MATLAB.

For double whole cell voltage clamp recordings cell pairs of ESdCs were selected and the whole cell configuration was established in both cells of the pair. Pipette and bath solutions were designed to minimize the transmembrane currents. The pipette solution consisted of (mM): Cs-Aspartate 115, TEA-Cl 20, EGTA 10, HEPES 10, Na_2ATP 5, pH adjusted to 7.3 with CsOH. Cells were superfused with a bath solution containing (mM): NaCl 146, $MgCl_2$ 0.5, $NiCl_2$ 6, $BaCl_2$ 1, CsCl 2, HEPES 5, glucose 5.5, pH 7.3 adjusted with NaOH. The total gap junctional current (I_j) of the cell pair was determined by inducing a voltage gradient (V_j) by holding cell 1 at 20 mV and cell 2 at 0 mV. The gap junction conductance was calculated as the I_j / V_j.

Electrophysiological Properties of HL-1 Cells and Monolayers

HL-1 cells express an atrial like phenotype. Current clamp recordings from individual cells within the monolayer revealed a minimum diastolic membrane potential of 71.3 ± 9 mV (n = 7) and atrial-like APs (Fig. S1A; APD_{50}= 51.3 ± 23 ms; overshoot = 9.1 mV; n = 7 room temperature). Heterogeneities within the cardiomyocyte monolayer could influence the ESdCs ability to become the dominant pacemaker in the ESdC/HL-1 co-cultures. We analyzed the homogeneity of HL-1 monolayers and their electrophysiological properties. HL-1 cells formed continuous monolayers that resumed spontaneous activity one day after plating. Due to the fact that the size of the HL-1 monolayer exceeded the area of the electrode field, the origin of excitation within the HL-1 monolayer was not identified. In monolayer preparations it is common that excitation starts at the border of the monolayer [3]. Electrical activity was synchronized

throughout the preparation (on all 60 MEA electrodes; Fig. S1B) and the monolayers developed reproducible beating frequencies of 4.3 ± 0.8 Hz and conduction velocities of 2.1 ± 0.4 cm/s (n = 70 cultures; 37^{o}C). The 2D-excitation spread within the culture obtained from the MEA recordings revealed evenly spaced contour plots (Fig. S1C), consistent with a homogeneous monolayer of excitable cells. Measurements of $[Ca^{2+}]_i$ in HL-1 monolayers that were loaded with the Ca indicator Fluo-4/AM [2] confirmed the continuity of excitation spread on the cellular level (Fig. S1DE). All cells along the line of the scan displayed excitation induced Ca-transients (Fig. S1E) with an average duration of 226 ± 35 ms at 50% amplitude (n = 26 cells at room temperature; Fig. 1F). The results underline that HL-1 cells form a homogeneous monolayer and therefore represent a suitable preparation to serve as the cardiac host tissue in our *in-vitro* pacemaker model.

Electrophysiological properties of WT- and Cx43(-/-)-ESdCs

We characterized structural, electrophysiological, and developmental parameters of WT- and Cx43(-/-)-ESdCs to identify the mechanism that enables Cx43(-/-)-ESdCs to gain and maintain pacemaker dominance at an increased rate. The area by which WT- and Cx43(-/-)-ESdCs interacted with the HL-1 monolayer exhibited no significant differences neither did the beating frequency of 14 day old WT- and Cx43(-/-)-ESdCs. A comparison of the developmental change of a WT-and Cx43(-/-)-ESdCs ISIs reveals the same time dependent decrease and excludes differences in the time courses of *in-vitro* differentiation. Double whole cell voltage clamp experiments confirmed that the lack of Cx43 expression results in a significant reduction of the Cx43(-/-)-ESdCs intercellular conductance; however, despite the change of ESdCs intercellular coupling no

significant change in the frequency variability (C_{IBI}) of the preparation could be determined [3].

[1] Sheehan KA, Blatter LA. Regulation of junctional and non-junctional sarcoplasmic reticulum calcium release in excitation-contraction coupling in cat atrial myocytes. J Physiol 2003;546(Pt 1):119.

[2] Kapur N, Banach K. Inositol-1,4,5-trisphosphate-mediated spontaneous activity in mouse embryonic stem cell-derived cardiomyocytes. J Physiol. 2007 Jun 15;581(Pt 3):1113-1127.

[3] Fahrenbach JP, Mejia-Alvarez R, Banach K. The relevance of non-excitable cells for cardiac pacemaker function. J Physiol. 2007 Dec 1;585(Pt 2):565-578.

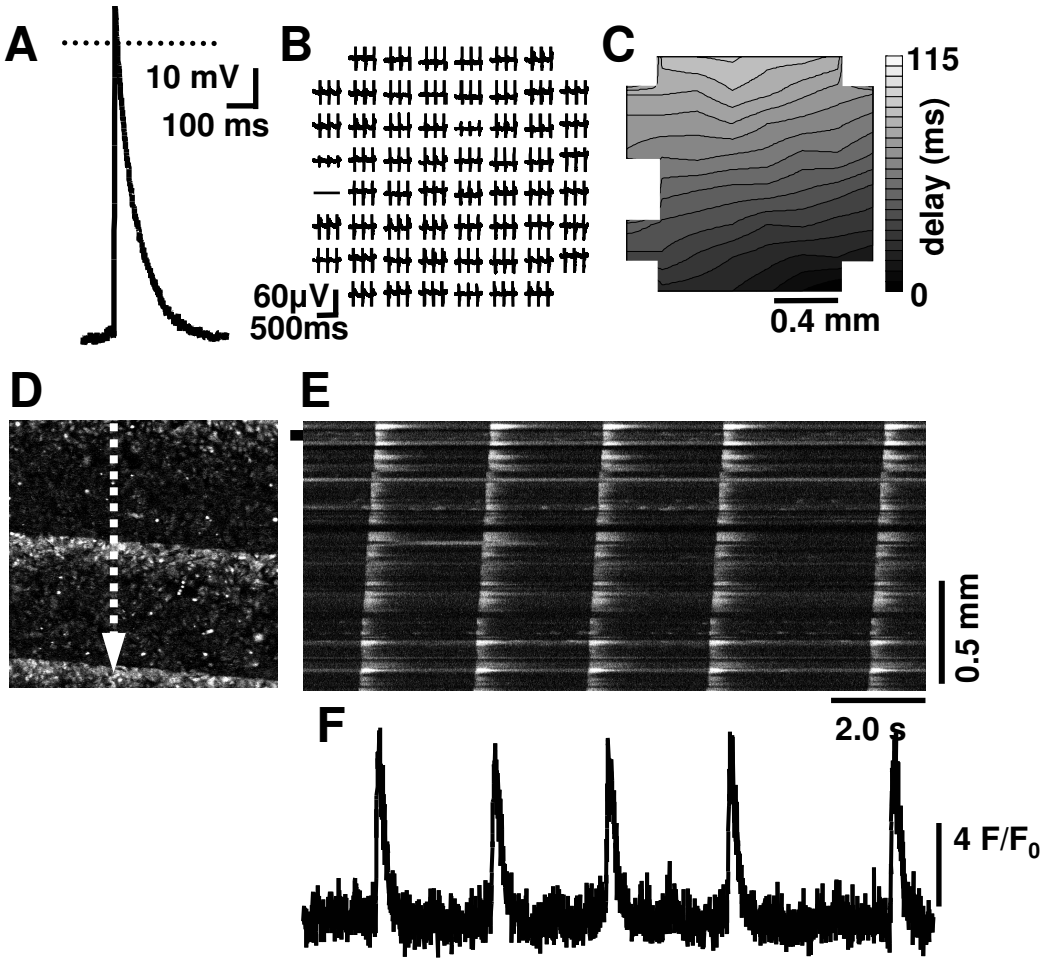

Figure S1: Electrophysiological properties of HL-1 cell monolayers. Current clamp recording (**A**) from a HL-1 cell displays an atrial-like AP upon stimulation (at room temperature: mean diastolic potential = -70.24 mV, overshoot = 8.95 mV; APD_{50} = 50.2 ms) (**B**) FP recordings from all 60 electrodes of the MEA (ground electrode left 4th from top) show synchronized electrical activity which translates into a contour plot (**C**) that shows continuous excitation spread. (**D**) xy-scan of a Fluo-4/AM loaded HL-1 monolayer. (**E**) The confocal line scan image (xt-scan from dotted line in **D**) shows excitation-induced Ca^{2+} transients in all cells along the line of scan. (**F**) Plot of the normalized fluorescence (F/F_0) over time shows the Ca^{2+} transients of an individual HL-1 cell (marked by the black box in panel **E**) within the monolayer.

Figure S1

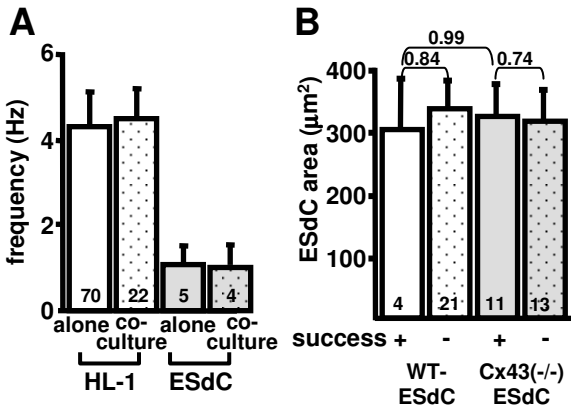

Figure S2: Beating frequency and size of transplanted ESdCs. (**A**) Beating frequency of HL-1 cells (white) and WT-ESdC aggregates (grey) in homocellular cultures (plain) or in co-cultures where either ESdC obtained pacemaker dominance (dotted). (**B**).Contact area of co-cultured WT- (white) and Cx43(-/-)-ESdC aggregates (grey) with the HL-1 monolayer depending on their success in establishing pacemaker dominance.

Figure S2

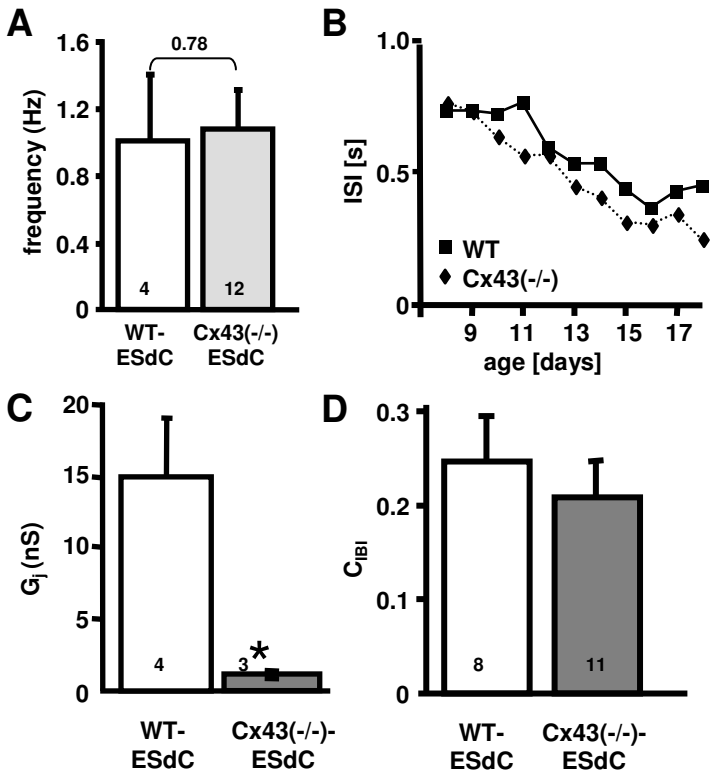

Figure S3: Electrophysiological properties of WT- and Cx43(-/-)-ESdC

(**A**) Average beating frequency of 14 to18 day old WT- and Cx43(-/-)-ESdC aggregates are not significantly different. (**B**) Representative examples of a WT- and a Cx43(-/-)-ESdC (not microdissected) for their differentiation dependent change in ISI on concomitant days in culture. (**C**) Gap junction conductance (G_j) of WT- or Cx43(-/-)-ESdC cell pairs. (**D**) The beating frequency variability (C_{IBI}) of transplanted WT- and Cx43(-/-)-ESdC aggregates. Number of experiments is specified within the bar; $*$ P> 0.05.

Figure S3