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Cellular and molecular mechanisms of arrhythmias in cardiac fibrosis and beyond : from symptoms to substrates towards solutions

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Cellular and Molecular Mechanisms of Arrhythmias in Cardiac Fibrosis and Beyond:
From Symptoms to Substrates towards Solutions

Chapter III

Connexin43 Silencing in Myofibroblasts Prevents Arrhythmias in Myocardial Cultures: Role of Maximal Diastolic Potential

Heterocellular Coupling and Arrhythmias

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Abstract

Aims: Arrhythmogenesis in cardiac fibrosis remains incompletely understood. Therefore, this study aims to investigate how heterocellular coupling between cardiomyocytes (CMCs) and myofibroblasts (MFBs) affects arrhythmogeneity of fibrotic myocardial cultures. Potentially this may lead to the identification of novel anti-arrhythmic strategies.

*Methods & Results***:** Co-cultures of neonatal rat CMCs and MFBs in a 1:1 ratio were used as a model of cardiac fibrosis, with purified CMC cultures as control. Arrhythmogeneity was studied at day 9 of culture by voltage-sensitive dye mapping. Heterocellular coupling was reduced by transducing MFBs with lentiviral vectors encoding shRNA targeting Connexin43 (Cx43) or Luciferase (pLuc) as control.

In fibrotic cultures, conduction velocity (CV) was lowered (11.2±1.6cm/s vs. 23.9±2.1cm/s $P<0.0001$), while action potential duration and ectopic activity were increased. Maximal diastolic membrane potential (MDP) of CMCs was less negative in fibrotic cultures. In fibrotic cultures, (n=30) 30.0% showed spontaneous reentrant tachyarrhythmias compared to 5% in controls (n=60). Cx43-silencing in MFBs made the MDP in CMCs more negative, increased excitability and CV by 51% ($P<0.001$), reduced action potential duration and ectopic activity ($P<0.01$), thereby reducing reentry incidence by 40%, compared to pLuc-silenced controls. Anti-arrhythmic effects of Cx43-downregulation in MFBs could be reversed by depolarization of CMCs through I_{k1} inhibition or increasing extracellular $[K^+]$.

Conclusion: Arrhythmogeneity of fibrotic myocardial cultures is mediated by Cx43 expression in MFBs. Reduced expression of Cx43 causes a more negative MDP of CMCs. This preserves CMC excitability, limits prolongation of repolarization and thereby strongly reduces the incidence of spontaneous reentrant tachyarrhythmias.

Introduction

Cardiac fibrosis is associated with an increased risk of potentially lethal tachyarrhythmias.[1] While increasing numbers of patients are suffering from such rhythm disturbances, the underlying cellular pro-arrhythmic substrate of fibrosis remains incompletely understood. In addition, there is a need for more effective treatment options.[2] This provides a strong incentive for obtaining a better understanding of the fibrotic substrate and to identify novel therapeutic targets.

One of the hallmarks of cardiac fibrosis is a substantial increase in the relative number of myofibroblasts (MFBs) that actively remodel the myocardium.[3,4] These MFBs are inexcitable, and may contribute to ectopic activity, conduction slowing and increased propensity towards reentrant tachyarrhythmias.[5-8] However, the exact mechanism through which MFBs exert their pro-arrhythmic effects remains incompletely understood. Various *in vitro* studies suggest that heterocellular coupling between cardiomyocytes (CMCs) and MFBs is potentially pro-arrhythmic. Through electrotonic interaction, CMC excitability is altered and conduction velocity (CV) and upstroke velocity are reduced while ectopic activity becomes more prevalent.[5] However, the effects of MFB-induced depolarization on repolarization dynamics remain largely unknown, especially how such alterations could be related to ectopic activity. Although heterocellular coupling has been proposed as a pro-arrhythmic mechanism, experimental down regulation of such coupling to establish its role in arrhythmogeneity has not been performed. To further define the pro-arrhythmic mechanisms in fibrotic myocardial cultures and to identify novel anti-arrhythmic targets, we investigated the effect of direct inhibition of MFB-CMC coupling on arrhythmogeneity of fibrotic myocardial cell cultures by selective down regulation of connexin43 (Cx43) expression in MFBs.

Our results demonstrate that inhibition of heterocellular coupling in co-cultures of CMCs and MFBs acts anti-arrhythmic by preserving CMC excitability and reducing prolongation of repolarization, both resulting from less MFB-induced depolarization. The maximal diastolic membrane potential (MDP) of CMCs was shown to be a key factor, as the anti-arrhythmic effects of MFB-selective Cx43 knockdown could be reversed by I_{k1} blockade-induced or high extracellular $[K^+]$-induced depolarization of CMCs.

Methods

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conform to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health. A more detailed description can be found in the Supplemental Material.

Cell isolation and culture

Primary ventricular CMCs and cardiac fibroblasts were isolated from hearts of neonatal Wistar rats. Animals were anesthetized using 4-5% isoflurane inhalation anesthesia. Adequate anesthesia was assured by absence of reflexes prior to rapid heart excision. Next, the ventricular cardiac tissue was chopped into small pieces and enzymatically digested under agitation at 37ºC by 2 sequential 45-minute treatments with collagenase type I (450 units/ml; Worthington, NJ, USA). The resulting cell suspension was transferred to plastic dishes (Falcon Primaria; BD Biosciences, Breda, the Netherlands) to allow preferential attachment of non-CMCs (mainly cardiac fibroblasts). Seventy-five minutes later, the non-adhered cells (mostly CMCs) were passed through a nylon cell strainer with a mesh pore size of 70 µm (Becton Dickinson, Franklin Lakes, NJ, USA) to remove undigested tissue fragments and cell aggregates. Next, the cells were counted and plated on fibronectin (Sigma-Aldrich, St. Louis, MO, USA)-coated round glass coverslips (15 mm Ø) at a density of 1-8x10^5 cells/well in 24-well plates (Corning Life Sciences, Amsterdam, the Netherlands), depending on the experiment.

Fibrosis was mimicked by co-cultures of primary CMCs and MFBs obtained from an earlier isolation. Cells were counted and mixed in a 1:1 ratio before plating, as this ratio has been shown to be pro-arrhythmic and allows for adequate detection of anti-arrhythmic effects.[8] To maintain initially seeded ratios (for fibrosis and control), all cultures were treated with the anti-proliferative agent mitomycin-C (Sigma-Aldrich, St. Louis, MO, USA) at day 1 of culture.[8] All cultures were refreshed daily with DMEM/Ham's F10 in a 1:1 mixture with 5% added horse serum (HS) (all from Invitrogen, Breda, the Netherlands) and cultured in a humidified incubator at 37ºC and 5% CO_2.

Immunocytological analyses

At day 9 of culture, cultures were stained for proteins of interest.[8] Quantification of fibroblast numbers was based on collagen type I staining.

Cultures were stained with antibodies specific for Cx43 (Sigma-Aldrich) or Cx45 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-actinin (Sigma-Aldrich), to study gap-junction formation between CMCs and MFBs. Corresponding AlexaFluor-

568 conjugated donkey-anti-mouse IgG and AlexaFluor-488 conjugated donkey-anti-rabbit IgG secondary antibodies (Invitrogen) were used at a dilution of 1:400. Nuclei were stained with Hoechst 33342 (10 µg/mL; Invitrogen). Fluorescent images were captured by fluorescent microscopy and analyzed using dedicated software (ImageJ, National Institutes of Health, Bethesda, MA, USA).

SIN-LV production

Vesicular stomatitis virus G-protein-pseudotyped self-inactivating human immunodeficiency virus type I vectors (SIN-LVs.) were produced in 293T cells. These cells were transfected with a lentiviral vector shuttle plasmid together with psPAX2 (Addgene, Cambridge, MA, USA) and pLP/VS.VG (Invitrogen) using 25-kDa linear polyethyleneimine (Polysciences, Warrington, PA, USA) as transfection agent. After 16 hours, transfection medium was replaced by culture medium and after 64 hours, the culture fluid was collected by centrifugation and freed of cellular debris by filtration. Concentration of lentiviral vector particles was performed by ultracentrifugation through a 20% (w/V) sucrose cushion. Pellets containing vector particles were suspended in phosphate-buffered saline with 1% bovine serum albumin fraction V (Sigma-Aldrich).

Lentiviral knockdown of Cx43 expression in MFBs

To suppress Cx43 expression, MFBs were transduced by lentiviral vectors carrying a Cx43-specific shRNA (Open Biosystems, Huntsville, AL, U.S.A.). The lentiviral vectors LV.SM2C.Cx43.hPGK.eGFP targeting Cx43 and LV.SM2C.pLuc.hPGK.eGFP targeting firefly luciferase (control) have been described previously.[9] Knockdown was structurally confirmed by immunocytological and Western Blot analyses and functionally confirmed by dye transfer assay.

Western blot analyses

At day 9 of culture, cultures were rinsed in ice-cold PBS and homogenized in RIPA-buffer. Homogenates were size-fractionated on NuPage 12% Bis-Tris gels (Invitrogen) before transfer to Hybond PVDF membranes (GE Healthcare, Leiderdorp, the Netherlands). These membranes were incubated with an antibody against Cx43 (Sigma-Aldrich) for 1 h followed by incubation with a matching HRP-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). To check for equal protein loading, β-tubulin (Millipore, Billerica, MA, USA) expression was determined. ECL Advance Detection reagents (GE Healthcare) were used to induce chemiluminescence which was subsequently caught on Hyperfilm ECL

(GE Healthcare). Intensities of Cx43 and β-tubulin bands were quantified by Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

Optical mapping

Action potential propagation patterns of purified CMC cultures and MFB-CMC co-cultures ($8x10^5$ cells/well in a 24-wells format) were studied by optical mapping with the voltage-sensitive dye di-4-ANEPPS (Invitrogen). On day 9 of culture, cells were incubated for 15±5 min with serum-free culture medium (DMEM/Ham's F10) containing 8 µmol/L di-4-ANEPPS. After incubation, cultures were refreshed with DMEM/Ham's F10 (37ºC) and subsequently mapped at 37ºC. To limit phototoxic effects, the same culture was never exposed to excitation light for ≥40s and mapping experiments typically did not exceed 30 min. Electrical activation was optically recorded (Ultima-L, SciMedia, Costa Mesa, CA, USA) and subsequently analyzed (Brain Vision Analyze 1103, Brainvision Inc, Tokyo, Japan). Action potential duration was defined as the period between the timepoint of maximal upstroke velocity and 90% repolarization (APD_{90}). Ectopic activity was defined as the presence of multiple sites of initiating activation.[8] Early afterdepolarizations (EADs) were defined as reversal of repolarization of at least 10% of the optical action potential amplitude of the initial activation, before full repolarization occurred. Pharmacological interventions were performed by directly pipetting the agent of interest into the mapping medium under mapping conditions, and effects were assessed after gentle agitation during an incubation period of 30 seconds in all groups. Tetrodotoxin (TTX) was obtained from Alomone Labs (Jerusalem, Israel).

Whole-cell patch-clamp

Whole-cell patch-clamp measurements were performed in purified CMC cultures, MFB cultures and co-cultures of CMCs and Cx43-silenced or pLuc-silenced, eGFP expressing MFBs at day 7-9 of culture. CMCs were identified by phase contrast microscopy and lack of fluorescence signal. Next, action potentials in CMCs were recorded in current-clamp at 25°C. Whole-cell recordings were performed using a L/M-PC patch-clamp amplifier (3 kHz filtering) (List-Medical, Darmstadt, Germany). Tip and seal resistance were 2.0-2.5 MΩ and >1 GΩ, respectively. For data acquisition and analysis, pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) was used. Parameters of interest were maximal diastolic potential (MDP), maximal upstroke velocity (dV/dt_{max}) and APD_{90}. In a different set of experiments, 0.5 mM $BaCl_2$ was added to the cultures to study the effect on MDP.

Dye transfer

Mitomycin-C-treated CMC cultures were loaded for 7 min with calcein-red-orange AM (Invitrogen) diluted in Hank's buffered salt solution (HBSS, Gibco, Grand Island, USA). Cells were rinsed twice and kept on culture medium containing 2.5 mmol/L probenecid (Invitrogen), which blocks multidrug resistance protein-dependent calcein efflux from the cells. Lentivirally eGFP-labeled MFBs or MFBs with Cx43 knockdown or pLuc knockdown were subsequently plated out in a 1:1 ratio with the calcein-loaded CMCs. The presence of dye transferred from CMCs to labeled MFBs and intensity of fluorescent signal were captured and subsequently quantified using dedicated software (ImageJ).

Statistical analysis

Statistical analyses were performed using SPSS11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were compared with one-way or two-factor mixed ANOVA test with Bonferroni post-hoc correction when appropriate, and expressed as mean±SD. Comparison between two groups was performed using paired or unpaired Student's-t test where appropriate. Differences were considered statistically significant if $P<0.05$.

Results

Conduction slowing, prolonged repolarization, ectopic activity and spontaneous reentrant tachyarrhythmias are commonly found in fibrotic cultures

In all cultures, >98% of all fibroblasts expressed α-SMA and collagen-I and were therefore considered MFBs as described previously.[8] In fibrotic cultures, relative MFB numbers were 53.0±0.6% at day 9, whereas purified CMC cultures contained 18.7±1.2% MFBs, as judged by quantification of collagen I/α-actinin staining (Figure 1A). To assess the arrhythmogenic potential of MFBs, fibrotic co-cultures of MFBs and CMCs and purified CMC cultures were assessed for several electrophysiological parameters using optical mapping. CV was significantly lower in fibrotic cultures compared to controls (11.2±1.6 cm/s vs. 23.9±2.1 cm/s; $P<0.0001$ n=30 and n=60) (Figure 1B). Basal spontaneous activation frequencies were 0.52±0.39Hz in controls and 0.90±0.61Hz in fibrotic cultures ($P<0.05$). Optical signal characteristics were distinctly altered in fibrotic cultures (Figure 1C). Time until 90% repolarization (APD_{90}), was significantly increased in fibrosis (Figure 1D). This signified slowing of repolarization in addition to slowed depolarization. Furthermore, prolonged repolarization was observed to precede early afterdepolarizations (EADs). EADs comprised 58% of 36 episodes of ectopic activity (Figure 1C arrow), whereas remaining episodes consisted of spatially alternating sites that initiated activation.

Consequently, of fibrotic cultures (total n=30), 53.3% showed ectopic activity as opposed to 18.3% of CMC cultures (total n=60) (Figure 1E, G). Moreover, the incidence of reentrant tachyarrhythmias was considerably higher in fibrotic cultures when compared to CMC cultures (5.0% vs. 30%) (Figure 1F, H). Of 14 episodes of spontaneous spiral wave formation, all were initiated by EADs, emphasizing their importance in arrhythmogenesis. Together, these results strongly demonstrate the pro-arrhythmogenic effects of MFBs in cardiac cultures.

Heterocellular coupling is associated with an elevated MDP of CMCs in fibrotic cultures

To study intercellular coupling between CMCs and MFBs, CMC-MFB co-cultures were stained for the gap-junctional proteins Cx43 and Cx45. Immunocytological staining revealed that Cx43 as well as Cx45 were expressed between adjacent MFBs, CMCs and MFBs adjacent to CMCs, suggesting the ability of MFBs to form homo- and heterocellular gap-junctions with both connexins (Supplemental Figure 1A). Of heterocellular junctions, 50.2% of 227 cell pairs showed expression of Cx43, whereas 25.5% of 180 cell pairs showed expression of Cx45. Furthermore, areas covered by Cx43 expression were significantly larger than areas covered by Cx45 at homocellular (Supplemental Figure 1B) and heterocellular junctions (Supplemental 1C), consistent with a dominant role of Cx43 in gap-junctional protein expression. Heterocellular gap-junction functionality was studied by calcein-red-orange dye transfer from CMCs to adjacent MFBs. Supplemental Figure 1D shows a representative image of adjacent calcein-positive MFBs and CMCs, confirming this ability, as isolated cells did not show such transfer of calcein (Supplemental Figure 1E). On an electrophysiological level, whole-cell current-clamp performed on CMCs in fibrotic cultures during spontaneous 0.5-1 Hz activation revealed a less negative MDP (-49.4±6.1 mV; n=7) compared to CMCs in control cultures (-64.8±5.9 mV; n=8; $P<0.001$) (Supplemental Figure 1F). The resting membrane potential of MFBs in homocellular cultures was -17.0±6 mV (n=9), which could explain their depolarizing effect on neighbouring CMCs after the establishment of functional heterocellular gap junctions.

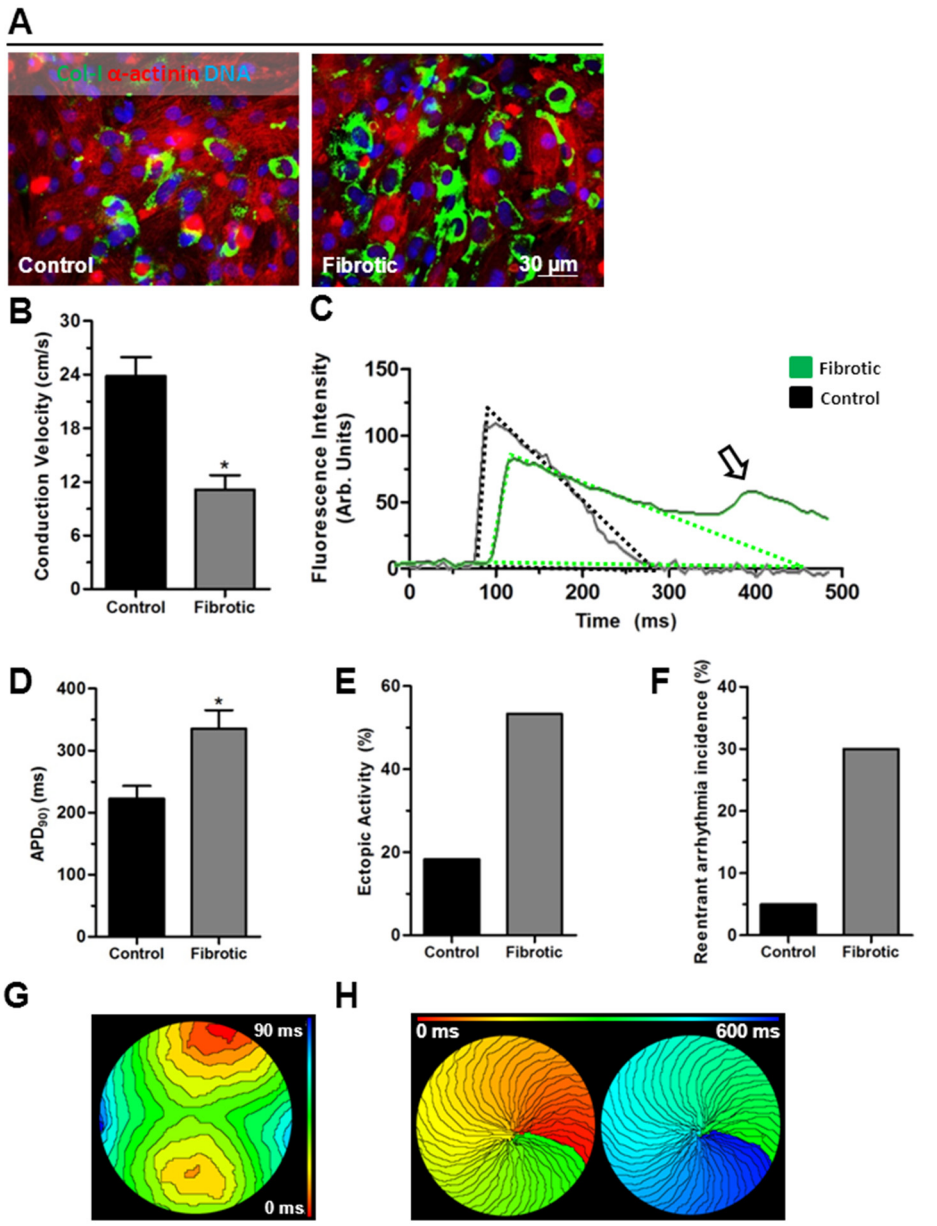
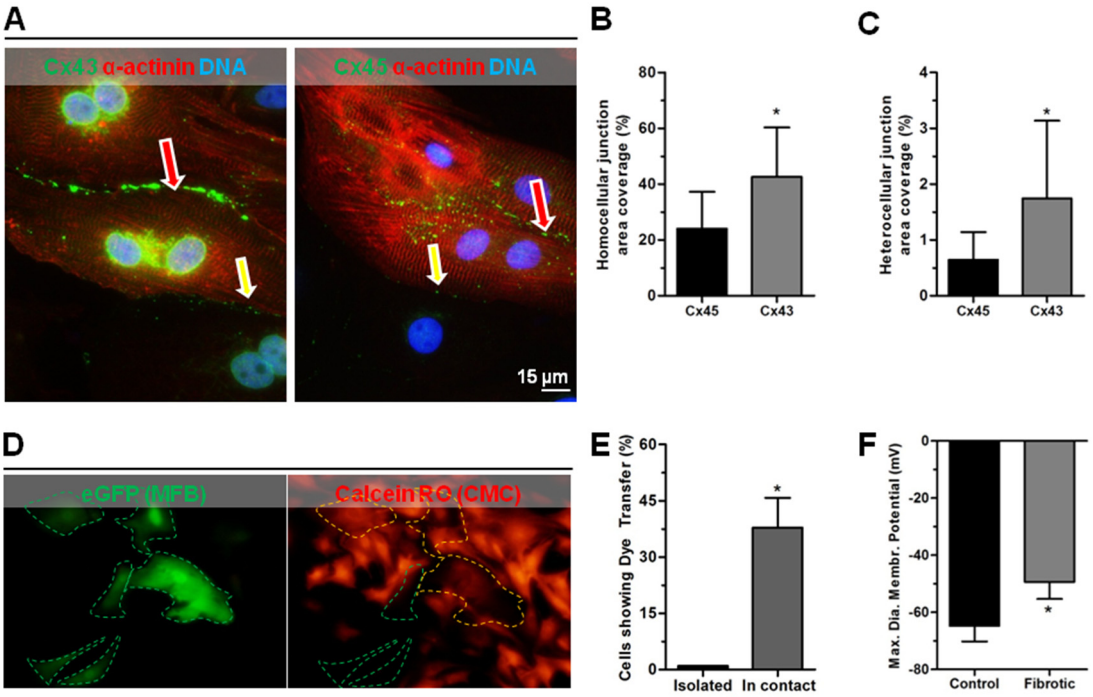


Figure 1. *Fibrotic cultures are pro-arrhythmic. (A) Typical example of a collagen I (green) and α-actinin (red) double staining showing more MFBs in fibrotic cultures compared to control cultures. Nuclei are stained blue with Hoechst 33342. (B) CV was slowed in fibrotic cultures *: P<0.001. (C) Typical optical signal trace shows prolongation of the action potential, the occurrence of an EAD in a fibrotic culture. (D) APD_{90} is increased in fibrotic culture. *:P<0.05*

vs. control. (E) Incidence of ectopic activity was significantly higher in the fibrotic group, as was the case for (F) spontaneous reentry. (G) Isochronal maps (6 ms) showing ectopic activity in a fibrotic culture and (H) 2 subsequent activations during a reentrant tachyarrhythmia in a fibrotic culture.



Supplemental Figure 1. Heterocellular coupling is associated with a less negative MDP of CMCs in fibrotic cultures*. (A) Cx43 and Cx45 were present at homo- and heterocellular junctions. Red arrows indicate homocellular CMC-CMC junctions. Yellow arrows indicate heterocellular MFB-CMC junctions. (B) Cx43 covers relatively larger areas at homocellular or (C) heterocellular junctions compared to Cx45. *p<0.05. (D) Typical photomicrographs of a dye transfer assay show that (E) eGFP-labeled MFBs in direct contact with calcein red-orange AM-loaded CMCs exhibit transfer of the calcein, whereas isolated MFBs showed no such transfer. (F) MDP obtained from whole-cell current-clamp recordings during 0.5-1 Hz spontaneous activity showed that CMCs in fibrotic cultures are significantly more depolarized as compared to those in purified CMC cultures (control). *: P<0.001.*

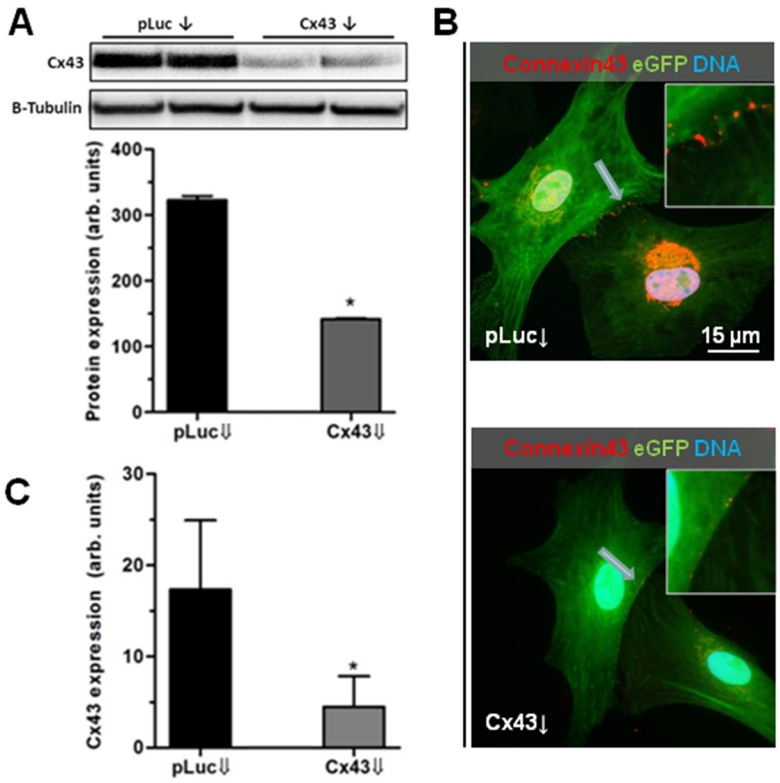
Taken together, these results implicate that MFBs and CMCs are functionally coupled and that CMCs are significantly depolarized in the proximity of MFBs, which may play a key role in the arrhythmogeneity of MFBs and may therefore represent a therapeutic target.

shRNA-mediated Cx43 silencing by viral transduction

To investigate heterocellular coupling as a potential target for anti-arrhythmic interventions, Cx43 expression in MFBs was downregulated by means of lentiviral shRNA-mediated RNA interference. After transduction with LV.SM2C.Cx43.hPGK.eGFP, Cx43 expression in MFBs was 53% lower than in LV.SM2C.pLuc.hPGK.eGFP-transduced MFBs as judged by Western blot analysis (Supplemental Figure 2A). Assessment of eGFP expression by fluorescence microscopy revealed that both SIN-LVs. had transduced the cells at equally high levels (Supplemental Figure 2B). To exclude the possibility that Cx43 knockdown solely affected the intracellular Cx43 pool, the western blot data were complemented with immunocytological analysis. After transduction with LV.SM2C.Cx43.hPGK.eGFP, Cx43 expression at MFB-MFB junctions was 74% lower in than control cells (Supplemental Figure 2B, C).

Silencing Cx43 expression in MFBs reduces functional heterocellular coupling without affecting homocellular CMC expression of Cx43

The effect of Cx43 knockdown on functional heterocellular coupling was first assessed by calcein transfer experiments. The effect of Cx43 knockdown on intercellular dye transfer between MFBs and CMCs was a lower percentage of calcein-positive LV.SM2C.Cx43.hPGK.eGFP-transduced MFBs in co-cultures when compared to Lv.SM2C.pLuc.hPGK.eGFP-transduced MFBs in co-cultures (22.4±6.5% vs. 40.8±10.6%; $P<0.01$ n=50 cell pairs across 7 photos per group) (Figure 2A and B). In addition, fluorescence intensity of the transferred dye was significantly lower in the Cx43 knockdown group than in the pLuc-silenced MFB group (Figure 2C). In 1:1 co-cultures with either pLuc- or Cx43-silenced MFBs, the MFB numbers and distribution were equal (Figure 2D). Homocellular Cx43 expression between CMCs was not affected by Cx43-silencing in MFBs. However, intercellular Cx43 expression at heterocellular junctions was 68% lower in co-cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs (2.7±1.3 arb. units vs. 8.5±3.8 arb. units; $P<0.001$, Figure 2E, F).



Supplemental Figure 2. *shRNA-mediated Cx43 silencing by lentiviral vectors decreases Cx43 expression in MFBs. (A) Typical example and quantification of Cx43 expression, normalized for β-tubulin expression from Western blot analysis showing significantly lower expression of Cx43 in Cx43-silenced MFBs compared to control (p<0.001). (B) Typical example of an immunocytological staining, showing less intercellular expression of Cx43 between Cx43-silenced MFBs compared to pLuc-silenced MFBs (control). (C) Quantification of intercellular Cx43 expression. *: p<0.001 vs. pLuc silencing (control).*

Preserved MDP and faster repolarization of CMCs in fibrotic cultures by heterocellular uncoupling

Next, CMCs in co-cultures were analysed by whole-cell patch-clamp to evaluate the electrophysiological consequences of Cx43 knockdown in fibrotic cultures (Supplemental Figure 3, Figure 3A). As a result of depressed heterocellular coupling, MDP was preserved in co-cultures with LV.SM2C.Cx43.hPGK.eGFP-transduced MFBs compared to co-cultures containing pLuc-silenced MFBs (-61.4±5.7 mV vs. -51.6±4.0 mV; *P*<0.05, (Figure 3B).Concomitantly, dV/dt_{max} was significantly higher in fibrotic

cultures with Cx43-silenced MFBs (Figure 3C), implying increased excitability. In addition, repolarization occurred faster in CMCs of the Cx43 knockdown group, as APD_{90} was reduced to 333±35 ms vs. 525±42 ms in the pLuc group (225±21 ms for non-fibrotic controls, Figure 3D).

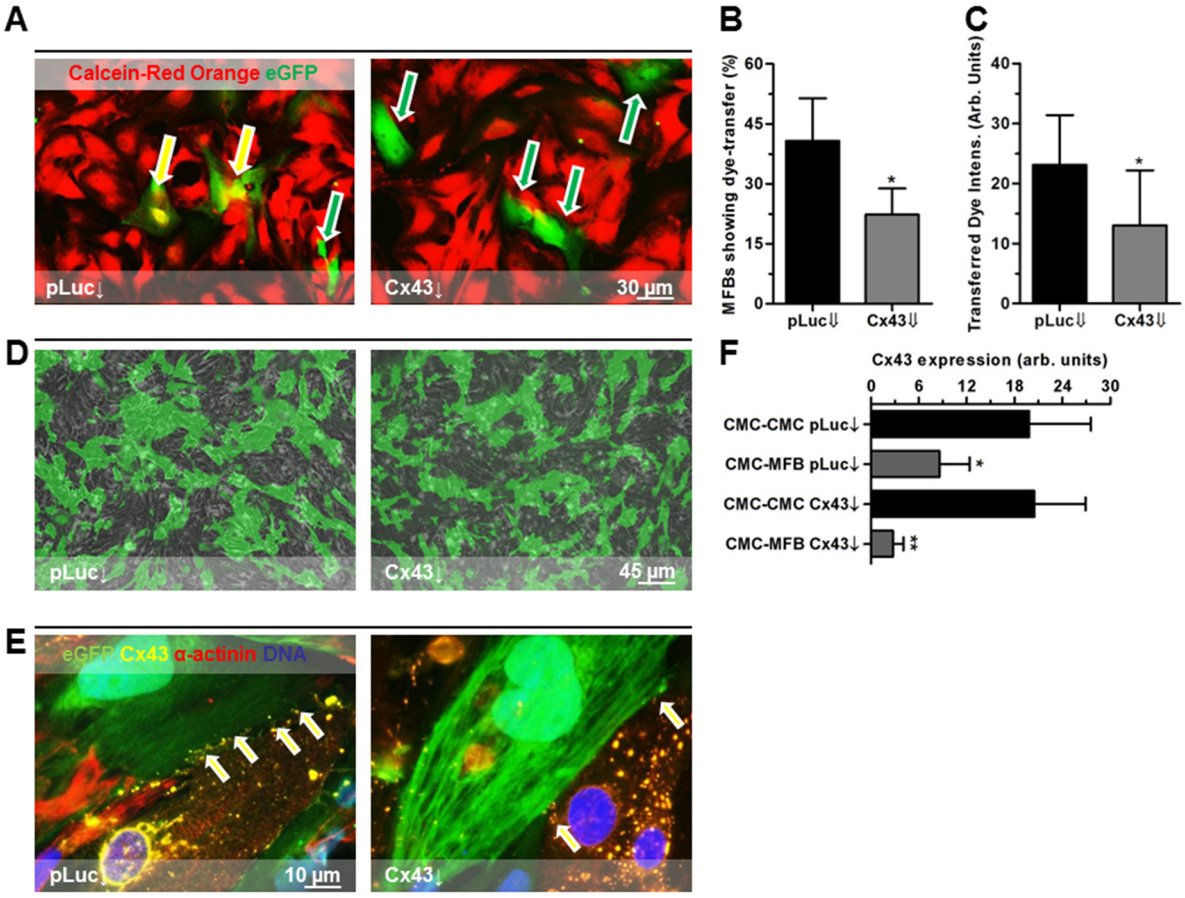
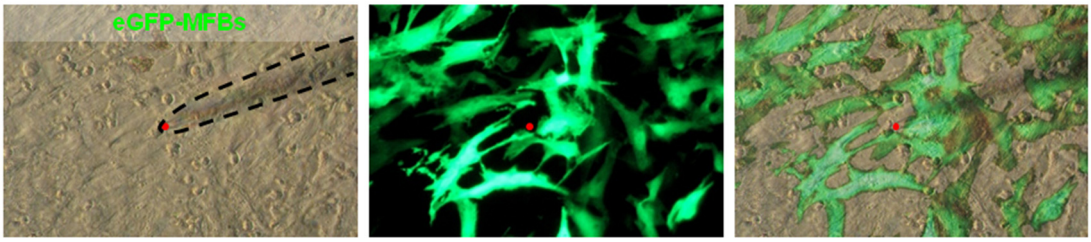


Figure 2. *Functional consequences of MFB Cx43 silencing in CMC-MFB co-cultures. (A) Typical examples of calcein Red-Orange loaded CMCs in co-culture with eGFP expressing, Cx43-silenced or pluc-silenced MFBs. Yellow arrows indicate MFBs that show transfer, whereas green arrows indicate MFBs that do not receive calcein from neighboring CMCs. (B) Quantification of the number of eGFP-positive MFBs showing transfer of calcein Red-Orange per image. *: P<0.001 (C) Fluorescence intensity of the transferred dye. *: P<0.001. (D) Typical examples of a merged image of phase-contrast and eGFP fluorescence showing equal distribution and relative numbers of Cx43-silenced or pLuc-silenced green MFBs in co-cultures. (E) Typical example of Cx43/α-actinin double staining showing decreased intercellular Cx43*

*expression in co-cultures with Cx43-silenced MFBs compared to co-cultures with pLuc-silenced MFBs (control). (F) Homo- and heterocellular Cx43 expression. *:P<0.05 vs. CMC-CMC pLuc silencing. **:P<0.05 vs. CMC-CMC Cx43 silencing and CMC-MFB pLuc silencing.*



Supplemental Figure 3. *EGFP-labeling of MFBs in fibrotic co-cultures allowed for the selective intracellular measurement of CMCs.*

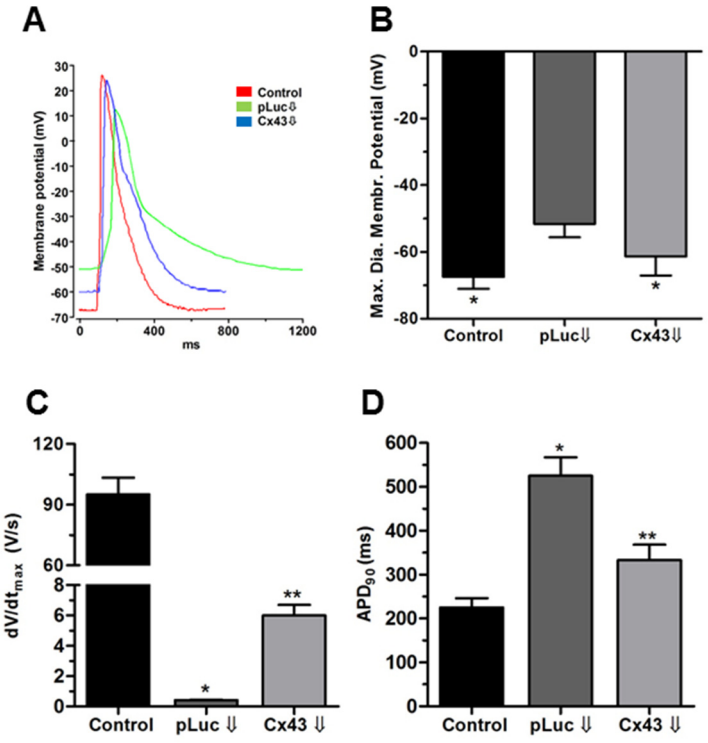


Figure 3. *Electrophysiological effects of Cx43 silencing in MFBs on CMCs at the intracellular level. (A) Typical examples of whole-cell patch-clamp recordings of CMCs in different myocardial cultures. (B) In fibrotic cultures with Cx43-silenced MFBs, the resting membrane potential of CMCs is significantly more negative as compared to CMCs in co-culture with pLuc-silenced MFBs, although not as negative as the resting membrane potential of CMCs in purified CMC cultures (control). *: P<0.05 vs. pLuc-silencing. (C) Maximal upstroke velocity in CMCs in co-culture with Cx43-silenced MFBs is significantly higher compared to CMCs in co-culture with pLuc-silenced MFBs. *:P<0.05 vs. control. **:P<0.05 vs. pLuc and Control. (D) APD_{90} is significantly decreased in fibrotic cultures with Cx43-silenced MFBs. *:P<0.05 vs. pLuc silencing.*

Cx43 knockdown in MFBs preserves excitability and prevents prolonged repolarization, ectopic activity and reentrant tachyarrhythmias in fibrotic myocardial cultures

At the tissue level, CV was significantly higher in co-cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs (16.0±3.2 cm/s vs. 10.6±4.3 cm/s; P<0.001) (Figure 4A, B). Furthermore, Cx43-silencing in MFBs resulted in morphological changes in optical action potentials (Figure 4C). Consistent with the dV/dt_{max} - increasing effect of MFB-specific Cx43 silencing in patch-clamp experiments, the decrease in CV after I_{na} inhibition by 20 µM TTX was significantly larger in co-cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs (Figure 4D). This indicated reduced MFB-induced depolarization and larger availability of Nav1.5 channels in CMCs by Cx43-knockdown in MFBs. This indicated reduced MFB-induced depolarization and larger availability of Nav1.5 channels in CMCs by Cx43-knockdown in MFBs.

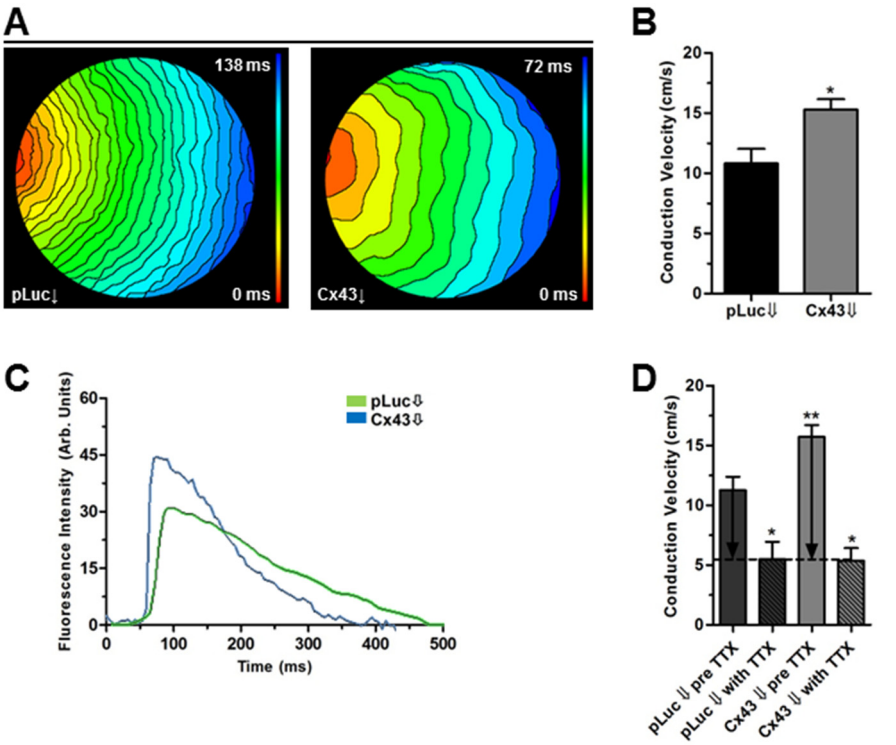


Figure 4. *Cx43-silencing in MFBs preserves culture excitability and fast conduction. (A) Typical activation maps of uniform propagation in co-cultures with Cx43-silenced MFBs and pLuc-silenced MFBs (6 ms isochrones). (B) CV was significantly higher for co-cultures with Cx43-*

*silenced MFBs than in co-cultures with pLuc-silenced MFBs. *: P<0.001 vs. fibrotic cultures with pLuc-silenced MFBs. (C) Optical signal traces show different action potential morphologies by Cx43-silencing in MFBs. (D) Nav1.5 blockade by 20 µM TTX decreases CV in both groups to similar values, although the decrease was larger for the Cx43-silenced group, indicating increased excitability. *:P<0.05 vs. pre TTX. **:P<0.05 vs. all.*

Findings of faster repolarization in patch-clamp experiments were also confirmed at the population level by optical mapping, as APD_{90} was significantly reduced by Cx43 knockdown in MFBs (Figure 5A, B, C). Furthermore, the incidence of ectopic activity in co-cultures was significantly decreased by Cx43 downregulation in MFBs. Of all co-cultures with Cx43-silenced MFBs 19.4% (total n=62) showed ectopic activity compared to 36.4% in co-cultures with pLuc-silenced MFBs (total n=33) (Figure 5D). Foremost, Cx43 knockdown in MFBs (n=55) decreased the incidence of reentrant tachyarrhythmias in co-cultures by 40% when compared to co-cultures with pLuc-silenced MFBs (n=32) (16.4% vs. 56.4%, Figure 5E). If a co-culture with Cx43-silenced MFBs exhibited a reentrant tachyarrhythmia, its reentrant cycle length was shorter than observed in a co-culture with pLuc-silenced MFBs (248±19ms vs. 296±48ms, *P*<0.05).

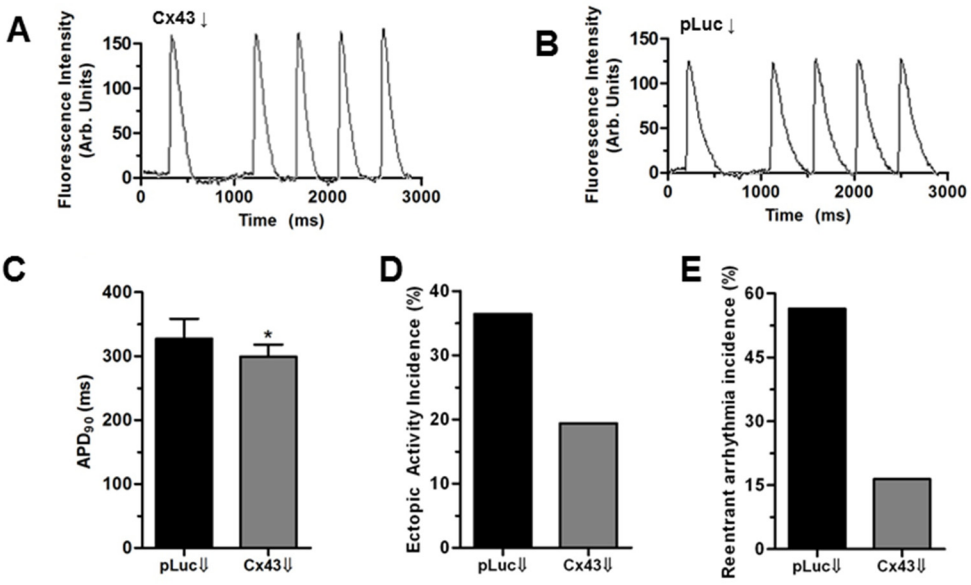


Figure 5. *Reduction of prolonged repolarization signifies a decrease in ectopic activity and spontaneous arrhythmias by Cx43-silencing in MFBs. (A) Typical optical signal trace during 1-2 Hz activation of fibrotic cultures with Cx43-silenced MFBs or (B) pLuc-silenced MFBs showing*

*that (C) APD_{90} was significantly decreased by Cx43-silencing in MFBs. *:P<0.05 vs. pLuc-silencing. (D) Co-cultures with Cx43-silenced MFBs showed less ectopic activity and (E) less spontaneous reentrant activity compared to co-cultures with pLuc-silenced MFBs (control).*

Re-induced depolarization abolishes anti-arrhythmic effects of Cx43 knockdown in MFBs in fibrotic myocardial tissue

To investigate whether the anti-arrhythmic mechanism of Cx43 knockdown was due to prevention of MFB-induced depolarization of the CMCs, depolarization was re-induced by I_{k1} inhibition with 0.5 mM $BaCl_2$. Indeed, $BaCl_2$ elevated the MDP of CMCs in co-culture with Cx43-silenced MFBs towards values found in the pLuc co-cultures (-59.0±4.7 mV (n=4) vs. -48.3±4.1 mV (n=4) before and after, respectively, P<0.05, Figure 6A). At the tissue level, this more positive MDP of CMCs caused by I_{k1} inhibition decreased CV in Cx43-knockdown cultures from 15.2±1.1 cm/s (n=62) to 9.7±1.1 cm/s (n=18), negating the higher CV normally found when compared to pLuc control cultures, which were not significantly affected (n=19, Figure 6B). Similarly, APD_{90} was increased in the Cx43 knockdown group after $BaCl_2$ (Figure 6C, D) and associated with increased ectopic activity by 27% (Figure 6E) and reentry by 36% (Figure 6F), thereby abolishing the anti-arrhythmic effects of down regulation heterocellular coupling in fibrotic myocardial cultures. To confirm these findings, CMCs in these cultures were depolarized by adding 10 mM of KCl to the mapping medium to increase the extracellular K^+ concentration to 14 mM. In accordance with the $BaCl_2$ experiments, increased extracellular $[K^+]$ also reversed the anti-arrhythmic potential of MFB-specific Cx43 knockdown in fibrotic co-cultures, as CV was decreased, whereas APD_{90} and ectopic and arrhythmic activity were increased in these cultures (Supplemental Figure 4).

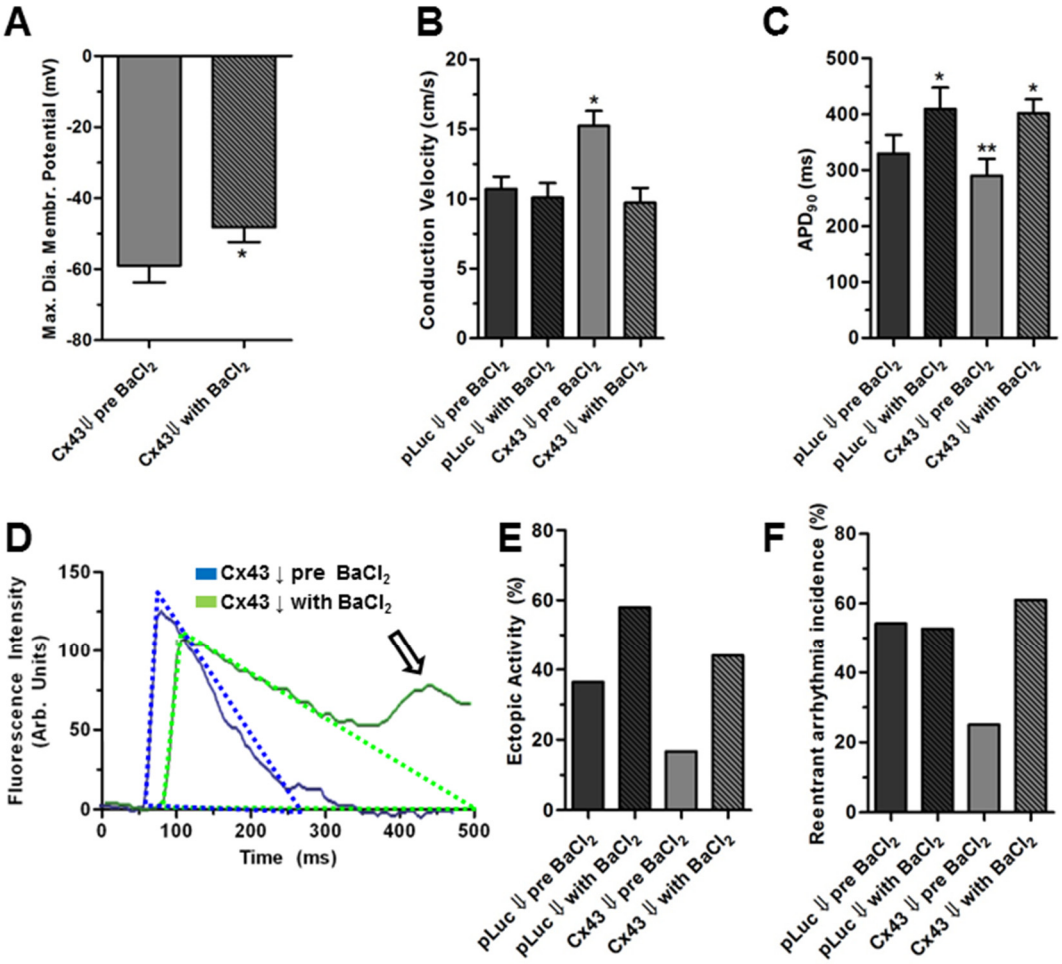
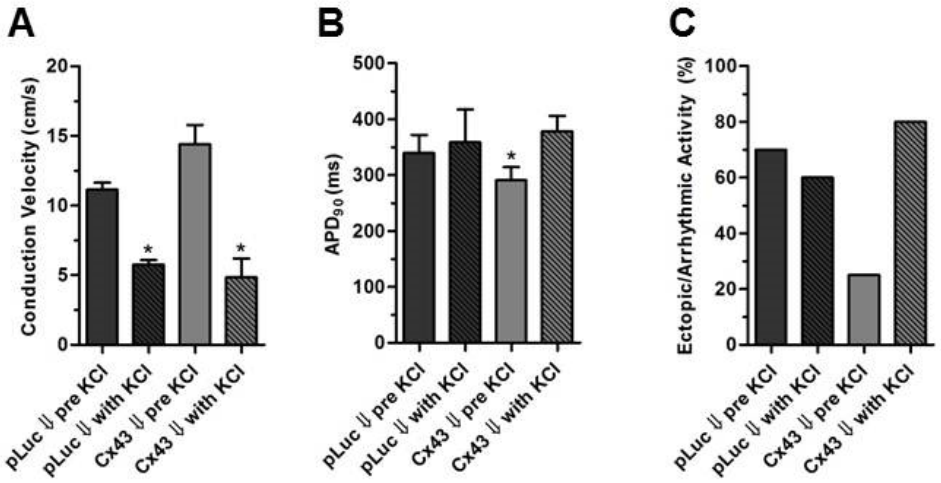


***Figure 6**. Increased excitability of CMCs after Cx43-silencing in MFBs and the preventive effect on ectopic activity and arrhythmias is reversed by depolarization of the resting membrane potential. (A) Inhibition of I_{k1} by 0.5 mM $BaCl_2$ elevated the MDP of CMCs in the Cx43-silencing group towards values of the pLuc group. The same intervention did not affect pLuc cultures. *:P<0.05 vs. all. (B) Depolarization by I_{k1} inhibition using BaCl2 slowed CV only in the Cx43 silencing group. *:P<0.05 vs. all. (C) I_{k1} inhibition cancels the effect of Cx43-silencing in MFBs on APD_{90} of optical action potentials and thus lengthens repolarization. (D) Increased APD_{90} by $BaCl_2$ increases the propensity towards EADs as marked by the arrow. (E) The preventive effect of Cx43 silencing on ectopic activity or (F) reentry is abolished by depolarization by I_{k1} inhibition.*



Supplemental Figure 4. *Depolarization in fibrotic myocardial cultures by increased extracellular K^+ reverses anti-arrhythmic potential of Cx43 knockdown in MFBs. Administration of 10 mM KCl to fibrotic CMC cultures has a more profound effect on (A) conduction velocity (*:p<0.05 vs pre KCl), (B) repolarization (*:p<0.05 vs all) or (C) ectopic and arrhythmic activity in co-cultures with Cx43-silenced MFBs than in those containing pLuc-silenced MFBs. The number of cultures analyzed were 10 and 10 for the pLuc group and 8 and 10 for the Cx43-knockdown group in the absence and presence of extra KCl, respectively.*

Discussion

Key findings of this study are (1) Silencing of Cx43 expression in MFBs leads to reduced heterocellular functional coupling and less MFB-induced depolarization of CMCs. 2) Preservation of the MDP of CMCs by Cx43 down regulation in MFBs leads to increased excitability and reduced prolongation of repolarization as compared to control, thereby limiting conduction slowing and decreasing ectopic activity. 3) The aforementioned changes are associated with a lower incidence of spontaneous reentrant tachyarrhythmias in fibrotic myocardial cultures. 4) MDP of CMCs is a key factor in the anti-arrhythmic effects of Cx43 down regulation in MFBs as all of the favorable changes could be reversed by depolarization of CMCs through I_{k1} inhibition or increasing extracellular $[K^+]$.

Cardiac fibrosis and abnormal impulse propagation and generation

The pro-arrhythmogenic effects of cardiac fibrosis, a process which consists of excessive MFB proliferation and extracellular matrix deposition, have been demonstrated *in vitro* and *in vivo*. Unfortunately, the exact pro-arrhythmic mechanisms are incompletely understood and the therapeutic efficacy of current anti-arrhythmic treatment options remains suboptimal. Initially, the mechanism by which cardiac fibrosis was considered to be pro-arrhythmic was that of extracellular matrix-mediated CMC separation, which leads to anatomical zigzag course of conduction and conduction block.[10,11] However, in recent years several studies have demonstrated that MFBs, independent of matrix deposition, could play a more crucial pro-arrhythmic role than initially thought. In fibrotic myocardial cultures, slow conduction, increased ectopic activity and propensity towards reentrant arrhythmias have been described, as was confirmed by the present study.[5-8]

In vitro studies have indicated that MFB-CMC coupling could be responsible for arrhythmogenesis by altering electrophysiological characteristics of CMCs. Functional heterocellular coupling between cardiac fibroblasts and CMCs was first observed by Rook *et al*.[12] The mechanism by which heterocellular coupling causes slowed conduction is thought to be based on electrotonic interaction leading to depolarization of CMCs to membrane potentials at which fast sodium channels are largely inactivated, thereby reducing excitability and CV.[13]

Besides causing conduction slowing, MFBs have also been shown to contribute to ectopic activity when seeded on top of cultured CMCs.[6] As the sarcolemma of CMCs becomes increasingly less negative with increasing numbers of adjacent MFBs, a level of depolarization can be reached that allows for depolarization-induced automaticity as basis for ectopic activity. Slowed conduction and ectopic activity are known to increase arrhythmogeneity in *in vitro* models of cardiac tissue and are both caused by heterocellular interactions.[5, 7, 14]

Anti-arrhythmic effects of Cx43 down regulation in MFBs

As electrotonic interaction is mediated through gap-junctions and has been suggested to be the basis of both conduction slowing and increased ectopic activity, this study investigated whether down regulation of this heterocellular coupling would have anti-arrhythmic effects. Although heterocellular coupling occurs through, at least, both Cx43 and Cx45-based connexons, a strong, functionally relevant reduction in heterocellular coupling could be achieved by solely down regulating Cx43 in MFBs. This can be explained by the higher expression levels of Cx43 at heterocellular junctions compared to Cx45, as was confirmed in this study.[14,15] The relatively higher occurrence of heterocellular Cx43 and Cx45

expression in this study compared to other studies can be explained by the notion that the fibroblastic cells are α-SMA positive myofibroblasts as opposed to α-SMA negative fibroblasts.[16] Conduction slowing by heterocellular coupling seems to be based on depolarization-induced inactivation of fast sodium channels, thereby reducing excitability. Indeed, the decrease in heterocellular coupling by lentiviral Cx43 knockdown in MFBs led to an increased CV and upstroke velocity. Nav1.5 blockade by 20 µM TTX yielded larger decreases in CV in the Cx43 knockdown group than in fibrotic control cultures, thereby proving that excitability was increased by reducing heterocellular coupling. Furthermore, by Cx43 knockdown in MFBs, CMCs were significantly less depolarized, also indicating increased excitability. As junctional Cx43 expression in MFBs was not completely abolished by transduction of these cells with LV.SM2C.Cx43.hPGK.eGFP, it was to be expected that CV would not be fully restored due to residual heterocellular coupling through Cx43 and Cx45. In addition, other mechanisms than heterocellular electrical coupling may contribute to arrhythmogeneity of fibrotic myocardial cultures. In a recent study, mechanical coupling and contractile forces between MFBs and CMCs were shown to affect CV and inducibility of arrhythmias in an anisotropic co-culture model.[17] However, the role of mechanical coupling may be of lesser influence in the current model, possibly because, unlike in the previous study, cells were not treated with TGF-β, different cellular ratios and tissue organizations were studied at different time-points.

Cx43 silencing in MFBs had also a pronounced effect on ectopic activity. The basis for increased ectopic activity in fibrotic cultures is still not fully understood. The current study provides a possible explanation by describing altered repolarization dynamics in fibrotic cultures. Triangulation of the action potential, or phase 3 prolongation is considered to be a highly pro-arrhythmic phenomenon as it can precede TdP *in vivo*.[18] In fibrotic cultures, APD_{90} was increased and preceded ectopic activity in the form of EADs. This is in accordance with computer simulations predicting that EADs occur more frequently in fibrotic cultures and are critical in spiral wave formation.[13,19] Prolongation of repolarization is currently thought to increase the chance of EAD generation by prolonging the time spent within the membrane potential range of the window current of L-type calcium channels, which allows for de-inactivation and subsequent reactivation.[20]

The combination of slowed conduction and increased ectopic activity is known to increase the pro-arrhythmic potential of cardiac tissue.[21] Hence, an increase in CV and a decrease in ectopic activity by Cx43 knockdown in MFBs reduce arrhythmogeneity, reflected by a lower incidence of spontaneous tachyarrhythmias. The importance of depolarization of CMCs as a key pro-arrhythmic mechanism was demonstrated by administration of $BaCl_2$, which effectively blocks I_{k1} and thereby

depolarizes CMCs independently of heterocellular coupling.[22] The dosage of $BaCl_2$ used in the present study is expected to completely and selectively block I_{K1} without significant effects on other potassium currents.[22,23] or L-type calcium current.[24] The I_{K1}-inhibition-induced depolarization immediately resulted in conduction slowing, prolonged repolarization, ectopic activity by EAD formation and spontaneous reentry in fibrotic cultures with Cx43-silenced MFBs, thereby reversing the anti-arrhythmic effect of reducing heterocellular coupling. In accordance, fibrotic cultures with pLuc knockdown as a control were not as strongly affected by $BaCl_2$-induced depolarization, suggesting that MFB-induced depolarization elevated the membrane potential towards values at which excitability was low enough to be relatively insensitive to further depolarization. Increasing extracellular $[K^+]$ confirmed the effects of $BaCl_2$ on reversing the anti-arrhythmic effect of Cx43-silencing in MFBs.
Taken together, the results of this study provide mechanistic insight into how MFBs exert their pro-arrhythmic effects on CMCs and how modulation of heterocellular coupling between CMCs and MFBs, as well as the MDP of CMCs could be targeted by anti-arrhythmic strategies.

Study limitations

Human adult CMCs are a more clinically relevant cell type than neonatal rat CMCs, but cannot be kept in culture for extended periods of time and are limited in availability. Although fibrosis consists of both an increase in MFBs and extracellular matrix, the latter was not investigated, as *in vitro* deposition of matrix of quality and quantity comparable to an in vivo situation is difficult to achieve. Although it is well established that MFBs and CMCs functionally couple *in vitro*, strong, undeniable proof of this phenomenon *in vivo* has yet to appear. Consequently, it is recognized that more *in vivo* research is necessary before *in vitro* results can be translated to clinical implications.

Conclusions

MFBs are able to form functional heterocellular gap-junctions with CMCs and are thereby able to diminish CMC excitability, reduce CV in cardiac cultures, prolong repolarization and induce ectopic activity, and give rise to spontaneous reentrant tachyarrhythmias in fibrotic myocardial cell cultures. Targeting heterocellular gap-junctional coupling by selective silencing of Cx43 expression in MFBs preserves CMC excitability, limits conduction slowing, prolonged repolarization and the incidence of ectopic activity and thereby prevents reentrant tachyarrhythmias. This effect resulted from decreased depolarization of CMCs through heterocelullar coupling as

I_{k1} inhibition or an increase in extracellular [K^+] abolished the anti-arrhythmic effects of Cx43-silencing in MFBs.

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Conflict of Interest
None declared

Supplemental Material

Methods

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conform to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

Immunocytochemical analyses

Following mapping experiments at day 9 of culture, cultures were stained for proteins of interest, or cultures were stained parallel to mapping experiments as described earlier.[1] Cultures were stained for α-smooth muscle actin (α-SMA) and vimentin expression to study fibroblasts phenotype (Sigma-Aldrich, St. Louis, MO, USA), collagen-I expression to quantify MFB numbers (Abcam, Cambridge, MA, USA), and α-actinin (Sigma-Aldrich) as CMC-specific marker. In addition, cultures were also stained for connexin43 (Cx43) (Sigma-Aldrich) and connexin45 (Cx45) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), to study gap junction formation between CMCs and MFBs. Corresponding AlexaFluor-568 conjugated donkey-anti-mouse IgG and AlexaFluor-488 conjugated donkey-anti-rabbit IgG secondary antibodies (Invitrogen, Breda, the Netherlands) were used at a dilution of 1:400. Nuclei were stained with Hoechst 33342 (10 µg/mL; Invitrogen). A fluorescent microscope equipped with a digital camera was used to capture images (Nikon Eclipse, Nikon Europe, Badhoevedorp, the Netherlands) and dedicated software (Image-Pro Plus, version 4.1.0.0, Media Cybernetics, Silver Spring, MD, USA) was used to analyze stained cultures. All proteins of interest were studied in at least 6 different cultures from a specific group, from which at least 20 representative images were acquired at various magnifications (10, 40, 100x). All cultures were stained using the same solutions and captured using equal exposure times for the protein of interest. Analysis of images was performed with ImageJ 1.44p (National Institutes of Health, USA). For analysis of gap-junctional Cx43 or Cx45 expression, percentual area coverage was determined in a fixed predefined area after setting a threshold value at 33% of maximal signal intensity. Fluorescent intensity levels were determined as an average pixel value within a fixed area, at least 5-fold per cell, or cell-cell junction for connexin staining.

SIN-LV production

To suppress Cx43 expression, MFBs were transduced by lentiviral vectors carrying a Cx43-specific shRNA (Open Biosystems, Huntsville, AL, U.S.A.). The lentiviral vectors LV.SM2C.Cx43.hPGK.eGFP targeting Cx43 and LV.SM2C.pLuc.hPGK.eGFP targeting firefly luciferase (control) have been described previously.[2]

Vesicular stomatitis virus G-protein-pseudotyped self-inactivating human immunodeficiency virus type I vectors (SIN-LVs.) were produced by seeding six 175 cm^2 culture flasks with 1 x 10^5 293T cells per cm^2 in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 10 µmol/L cholesterol (Sigma-Aldrich). The next day, these producer cells were transfected with either one of the lentiviral vector plasmids together with psPAX2 (Addgene, Cambridge, MA, USA) and pLP/VSVG (Invitrogen) at a 2:1:1 molar ratio using a total of 200 ng DNA/cm^2 and 3 ng polyethyleneimine (Polysciences, Warrington, USA) per ng DNA as transfection agent. Sixteen hours later, the transfection medium was replaced by DMEM containing 5% FBS, 10 mM HEPES-NaOH (pH 7.4) and 10 µmol/L cholesterol. At 64 h post-transfection, the culture fluid was collected and freed of cellular debris by centrifugation at room temperature for 10 min at 825x *g* and filtration through a 0.45 µm pore-sized cellulose acetate filter (Pall Corporation, East Hills, NY, USA). To concentrate the lentivirus vector particles, a 5 mL cushion of 20% sucrose in phosphate-buffered saline (PBS) was loaded under 30 ml of the cleared culture medium, which was then centrifuged for 90 min at 15,000 rpm and 10°C in an SW28 rotor (Beckman Coulter, Fullerton, CA, USA). Next, the supernatant was discarded and the pellets with the vector particles were suspended in 400 µL of PBS containing 1% bovine serum albumin (BSA fraction V from Sigma-Aldrich) by gentle rocking overnight at 4°C.

The gene transfer activity of the vector stocks was determined by end-point titration on HeLa indicator cells using flow-cytometric analysis of *eGFP* as read-out. The titers of the SIN-LV preparations are thus expressed in HeLa cell-transducing units (HTUs) per mL.

MFBs were transduced with SIN-LV particles at a multiplicity of infection (MOI) of 32 HTUs per cell in culture medium containing 20 µg/mL diethylaminoethyl-dextran sulfate (GE Healthcare, Diegem, Belgium). After 4 h, the cultures were washed three times with PBS and supplied with fresh culture medium. MFBs were passaged twice before application in experiments.

Western blot analyses

MFB cultures were transduced with LV.SM2C.Cx43.hPGK.eGFP or LV.SM2C.pLuc.hPGK.eGFP parallel to mapping experiments. At day 9 of culture, transduced and mock-transduced cultures were rinsed in ice-cold PBS and homogenized in RIPA-buffer consisting of 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP-40, 0.5% sodiumdeoxycholate, and 0.1% SDS. Homogenates were size-fractionated on NuPage 12% gels before wet transfer to Hybond PVDF membranes (GE Healthcare). These membranes were incubated with an antibody against Cx43 (Sigma-Aldrich) for 1 h followed by incubation with corresponding HRP-conjugated secondary antibody (Santa Cruz). To check for equal protein loading, β-tubulin (Millipore, Billerica, MA, USA) expression was determined. ECL Advance Detection reagents (GE Healthcare) were used to induce chemiluminescence which was subsequently caught on Hyperfilm ECL (GE Healthcare). The intensity of Cx43 and β-tubulin-specific signals were quantified by Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

Optical mapping

Action potential propagation patterns of purified CMC cultures and CMC-MFB co-cultures ($8x10^5$ cells/well in 24-wells plate) were studied by optical mapping with the voltage-sensitive dye di-4-ANEPPS (Invitrogen). Structurally heterogenous cultures (<5%, determined by light microscopy or electrophysiological mapping) were all excluded for reasons of standardization and reproducibility. On day 9 of culture, cells were incubated for 15±5 min with culture medium (1:1 DMEM/Ham's F10 + 5% HS) containing 8 µmol/L di-4-ANEPPS. After incubation, cultures were refreshed with DMEM/Ham's F12 (37ºC) and subsequently mapped at 37º C. Epi-illumination excitation light (λ_{ex}=525±25 nm) was delivered by a halogen arc-lamp (MHAB-150W, Moritex Corporation, San Jose, CA, USA). To limit phototoxic effects of the mapping protocol on the cultures, the same culture was never exposed to excitation light for longer than 40s and mapping experiments in a 24-well plate typically did not exceed 30 min. Fluorescent emission light (λ_{em}>590 nm) passed through a camera lens (1x Plan-Apo, WD=15 mm; Leica, Wetzlar, Germany) and a dichroic mirror, after which it was focused onto a 100 by 100 pixels (100 mm^2) CMOS camera (Ultima-L, SciMedia, Costa Mesa, CA, USA) by a 1.6x converging lens, resulting in a total field of view of 256 mm^2 and a spatial resolution of 160 µm/pixel. Electrical activation was recorded for at least 4 s at a rate of 167 or 500 frames/s, high-pass filtered and analyzed using Brain Vision Analyze 1103 (Brainvision Inc, Tokyo, Japan). For each pixel, signals were averaged with 8 of its nearest neighboring pixels. Time point at which the rate of rise of fluorescence signal (d*F*/dt) was maximal was defined as the activation time point.

CV in cultures with a uniform activation pattern at an activation frequency of 1-2 Hz, was calculated between two 3 by 3 pixel grids, typically spaced 2-8 mm apart, and perpendicular to the activation wavefront. Per culture, CV was determined in 6-fold and averaged for further comparisons. Action potential duration, measured from the timepoint of maximal upstroke velocity (dF/dT_{max}) until 90% of repolarization (APD_{90}), was determined in 3-fold and averaged. Reentry was defined as >4 cycles of a circular activation pattern. Reentrant cycle length was calculated from 3 separate cycles per culture and averaged. Ectopic activity was defined as the presence of multiple sites of initiating activation.[1] Early after depolarizations (EADs) were defined as the reversal of repolarization of at least 10% of the optical action potential amplitude of the previous activation. Pharmacological interventions were performed by directly pipetting the agent of interest into the mapping medium under mapping conditions and effects were assessed after gentle agitation during an incubation period of 30 seconds in all groups.

Dye transfer

To investigate functional coupling between MFBs and CMCs, eGFP-labeled MFBs were kept in culture for 2 weeks and at least passaged twice before they were used in experiments. CMC cultures containing 10^5 cells/well were treated with 10 µg/ml mitomycin-C to prevent endogenous MFB overgrowth. At day 4, these cultures (n=12) were loaded for 7 min with 10 µg/mL calcein-red-orange AM (Invitrogen) in HEPES-buffered salt solution (HBSS, Gibco, Grand Island, USA), which once internalized is hydrolyzed to the orange fluorescent dye calcein-red-orange. Cells were rinsed twice with PBS and kept on culture medium containing 2.5 mmol/L probenecid (Invitrogen) which prevents calcein efflux[25]. eGFP-labeled MFBs were subsequently plated out in a 1:1 ratio with the calcein-loaded CMCs. To determine the functional effect of Cx43 downregulation in MFBs, dye transfer experiments were performed using MFBs transduced with LV.SM2C.Cx43.hPGK.eGFP or LV.SM2C.pLuc.hPGK.eGFP, and CMCs loaded with 10 µg/mL Calcein Red-Orange-AM (Invitrogen). Fluorescent images (at least 10 per culture, at least 12 cultures per group) were acquired after 7 h of co-culture under equal exposure times and magnifications. The percentage of eGFP-labeled MFBs that had received calcein from adjacent CMCs as well as the intensity of the dye-associated fluorescent signal per calcein-positive MFB were determined using ImageJ.

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