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 II

Antiproliferative Treatment of Myofibroblasts Prevents Arrhythmias *In Vitro* by Limiting Myofibroblast-Induced Depolarization

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Abstract

Aims: Cardiac fibrosis is associated with increased incidence of cardiac arrhythmias, but the underlying pro-arrhythmic mechanisms remain incompletely understood and antiarrhythmic therapies are still suboptimal. This study tests the hypothesis that myofibroblast (MFB) proliferation leads to tachyarrhythmias by altering the excitability of cardiomyocytes (CMCs), and that inhibition of MFB proliferation would thus lower the incidence of such arrhythmias.

Methods & Results: Endogenous MFBs in neonatal rat CMC cultures proliferated freely, or under control of different dosages of antiproliferative agents (mitomycin-C and paclitaxel). At day 4 and 9, arrhythmogeneity of these cultures was studied by optical and multi-electrode mapping. Cultures were also studied for protein expression and electrophysiological properties.

MFB proliferation slowed conduction from 15.3±3.5 cm/s (day 4) to 8.8±0.3 cm/s (day 9) (n=75, $P<0.01$), while MFB numbers increased to 37.4±1.7% and 62.0±2%. At day 9, 81.3% of these cultures showed sustained spontaneous reentrant arrhythmias. However, only 2.6% of mitomycin-C treated cultures (n=76, $P<0.0001$) showed tachyarrhythmias, and ectopic activity was decreased. Arrhythmia incidence was drug-dose dependent and strongly related to MFB proliferation. Paclitaxel-treatment yielded similar results. CMCs were functionally coupled to MFBs, and more depolarized in cultures with ongoing MFB proliferation, in which only L-type Ca^{2+} channel-blockade terminated 100% of reentrant arrhythmias, in contrast to Na^{+}-blockade.

Conclusion: Proliferation of MFBs in myocardial cultures gives rise to spontaneous, sustained reentrant tachyarrhythmias. Antiproliferative treatment of such cultures prevents the occurrence of arrhythmias by limiting MFB-induced depolarization, conduction slowing and ectopic activity. This study could provide a rationale for a new treatment option for cardiac arrhythmias.

Introduction

Cardiac arrhythmias remain a leading cause of mortality in the Western world, despite a variety of treatment options.[1] Particularly, implantable cardioverter defibrillators have shown to be effective in improving survival of patients at risk. However, the underlying arrhythmogenic substrate is left untreated and therefore the occurrence of arrhythmias is not prevented.[2] Catheter ablation therapy may serve as an alternative and potentially curative treatment modality. However, its long-term benefits and effects on survival are yet unknown.[3] Furthermore, anti-arrhythmic drug therapy appears to have no significant effect on the survival in larger groups of patients suffering from cardiac arrhythmias and is associated with significant and potentially lethal side effects.[4] The limited therapeutic efficacy and adverse effects associated with these therapies is partly explained by our insufficient understanding of the tissue substrate and pro-arrhythmic mechanisms that are responsible for the occurrence of lethal ventricular tachyarrhythmias.

Taken together, current treatment of cardiac arrhythmias, including means to prevent arrhythmias, is still suboptimal. It is therefore essential to better comprehend the underlying pro-arrhythmic tissue substrate and to provide new rationales for the development of more effective treatment options aimed at preventing arrhythmias from occurring.

Cardiac fibrosis, for example as a result of ischemic heart disease and aging, deteriorates the well-organized nature of the working myocardium due to a dramatic increase in fibroblastic cells, called myofibroblasts (MFBs).[5,6] This may increase electrical heterogeneity and the risk for lethal arrhythmias.[7,8] However, the functional role of MFBs in cardiac arrhythmias is still incompletely understood, especially the impact of their proliferative capacity on myocardial tissue. We hypothesized that MFB proliferation is a key factor in the incidence of spontaneous arrhythmias by altering the excitability of cardiomyocytes (CMCs), resulting in slow conduction and increased ectopic activity, and that inhibition of MFB proliferation may lower, or even prevent, the incidence of cardiac arrhythmias. To test this hypothesis, we studied the role of MFB proliferation in the occurrence of spontaneous reentrant arrhythmias in cardiac cultures using several antiproliferative agents, with cytological and extra- and intracellular electrophysiological techniques.

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, cell culture and antiproliferative treatment

Neonatal rat ventricular CMCs were isolated and cultured as described previously.[9] Cells were plated on fibronectin-coated, round coverslips (15 mm) at a density of 4-8x10^5 cells/well in 24-well plates. Endogenous MFBs, present in these cultures, were allowed to proliferate freely, or under control of antiproliferative agents (mitomycin-C (0.05-10 µg/ml) and paclitaxel (0.085 mg/ml), which were added at day 1 of culture and incubated for 2h, resulting in partial or full inhibition of MFB proliferation. In addition, defined ratios of MFBs and CMCs (10/90%, 25/75%, 50/50%) were mixed and co-cultured in 24-well plates and treated with mitomycin-C as described above, to maintain initial ratio and cell density.

Immunocytological analyses

Cultures were fixed in 1% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with 1:50-1:200 diluted primary antibodies (see Online Supplement for details on antibodies). Corresponding Alexa fluor-conjugated secondary (Invitrogen, Carlsbad, CA, USA) antibodies were used at a dilution of 1:400. Subsequently, nuclei were counterstained with Hoechst 33342. Cultures were photographed and quantified with dedicated software (Image-Pro Plus, version 4.1.0.0, Media Cybernetics, Silver Spring, MD, USA).

Western blot analyses

Homogenates were made from either 3 different purified CMC cultures, 50/50% CMC/MFB co-cultures or purified MFB cultures. Next, proteins were separated by SDS-page and transferred to Hybond PVDF membranes. Blots were blocked in 5% bovine serum albumin in TBS-T. Primary and corresponding HRP-conjugated secondary antibodies were incubated for 1 h, after which chemiluminescence was induced by ECL advance detection reagents.

Proliferation assays

Proliferation assays consisted of quantification of Ki67 expression as judged by Ki67 staining. Furthermore, MFB numbers in cardiac cultures were quantified at day 1, 4 and 9, based on collagen-I staining.

Apoptosis assay
Possible pro-apoptotic effects of the antiproliferative treatments were investigated by active caspase-3 staining, using aforementioned protocol.

Optical and multi-electrode mapping
At day 4 and 9, cardiac cultures were loaded with 16 μmol/L di-4-ANEPPS, given fresh DMEM/Ham's F12 (37ºC) and immediately mapped using the Ultima-L optical mapping setup (SciMedia, Costa Mesa, CA, USA). Throughout mapping experiments, cultures were kept at 37ºC. Optical signal recordings were analyzed using Brain Vision Analyze 0909 (Brainvision Inc, Tokyo, Japan) in order to assess conduction velocity (CV). Spontaneous ectopic activity was assessed in all groups for 24 s after unipolar electrical stimulation, so that if present, reentrant arrhythmias were eliminated, which allowed for ectopic or other spontaneous activity to resume.
For multi-electrode array (MEA) mapping, cells were cultured in glow-discharged, fibronectin-coated MEA culture dishes (Multi Channel Systems, Reutlingen, Germany) and measurements were performed in the associated data acquisition system, typically within 10 seconds after optical mapping. Electrograms were analyzed off-line using MC-Rack software (version 3.5.6, Multi-Channel Systems).

Whole-cell patch clamp and dye transfer
Measurements were performed in co-cultures of CMCs and MFBs treated with or without mitomycin-C at day 9 of culture, or co-cultures of CMCs and eGFP-labeled MFBs. After identification of CMCs by phase contrast or fluorescence microscopy, maximal diastolic potentials in CMCs were recorded in current-clamp. For data acquisition and analysis, pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) was used. To further study functional cell-cell coupling, co-cultures of calcein AM-loaded CMCs (green) and Katushka-expressing MFBs (red) were investigated for gap junction-mediated calcein transfer into MFBs by fluorescence microscopy.

Pharmacological interventions
The role of ion channel blockade in the maintenance of reentrant arrhythmias was investigated using the selective Nav1.5 blocker Tetrodotoxin (TTX, 5-10 μmol/L; TTX, Sigma-Aldrich) or verapamil (100 μmol/L; Centrafarm, Etten-Leur, the Netherlands) as Cav1.2 blocker. These blockers were added to the mapping medium, after which the cultures were studied by optical mapping.

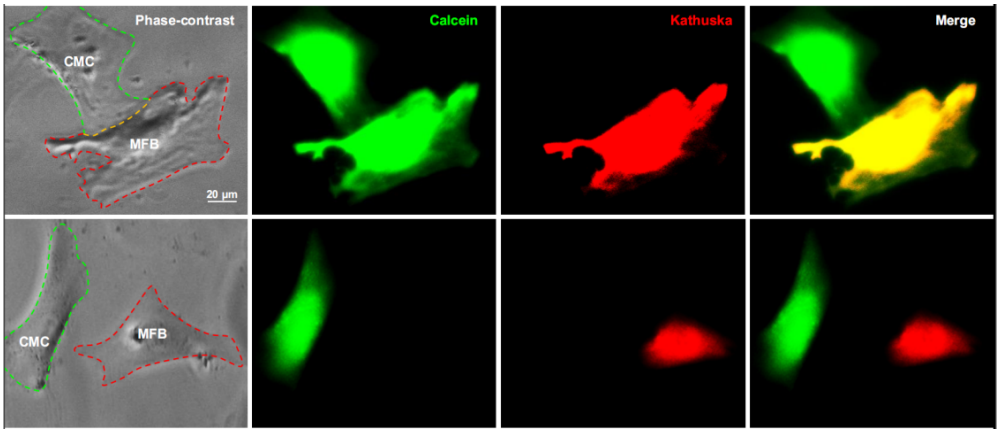
Statistical analyses

Statistical analyses were performed using SPSS11.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant if $P<0.05$. A more detailed description of the Materials and Methods can be found in the Online Supplement.

Results

Characterization of cardiac cell cultures

Cultures from neonatal rat ventricles (25 isolations) were studied for expression of cell type-specific and gap junction proteins at day 9 of culture. All cultured fibroblasts had the MFB phenotype as judged by α-smooth muscle actin (α-SMA) and vimentin expression (Figure 1). Connexin43 (Cx43) was present between adjacent CMCs, MFBs and at heterocellular junctions (Figure 1A).Dye transfer experiments demonstrated functional gap junctional MFB-CMC coupling (supplemental Figure 1). Western blot analyses revealed an inverse linear relationship between MFB percentage and Cx43 levels. In contrast, α-SMA levels showed a positive linear relationship with MFB numbers (Figure 1B-C). Of all α-SMA positive MFBs, 98.5±1.6% also expressed cytoplasmic collagen-I, centered at the nucleus (R^2=0.9921) (Figure 1D, G). CMCs did not express collagen-I, but stained positive for α-actinin (Figure 1E). Of the vimentin-positive MFBs, 98.4±1.3% also co-expressed collagen-I (R^2=0.9960) (Figure 1F, H). Immunocytological staining for collagen-I as MFB marker and cardiac α-actinin as CMC-specific marker therefore allowed us to quantify endogenous MFBs in a reliable and standardized manner.



Supplemental Figure 1. *Dye transfer experiments of CMCs loaded with calcein (green) and MFBs labeled with Kathushka (red). MFBs in direct contact with calcein-loaded CMCs (top) were positive for the gap-junctional permeable green fluorescent dye calcein. In contrast, MFBs that remained separate (bottom) from CMCs were negative for this dye. These results indicate that CMCs and MFBs are functionally coupled.*

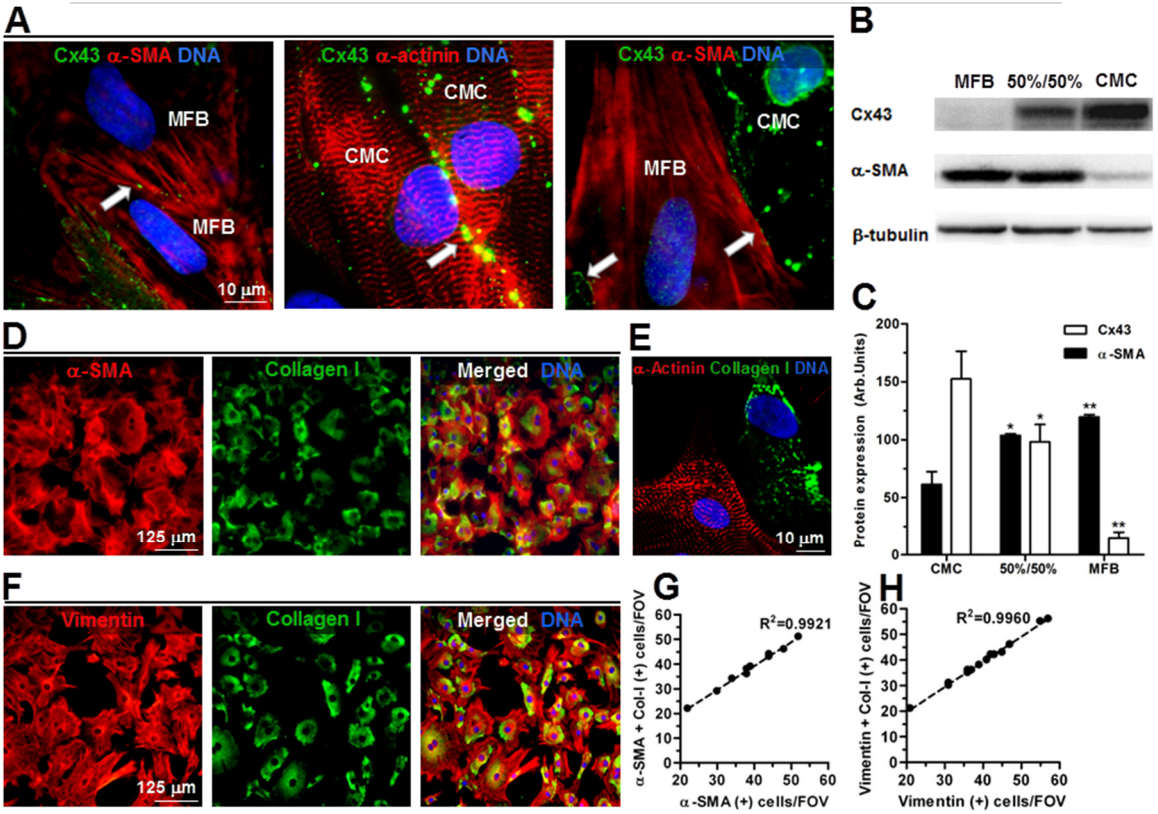


Figure 1. *Characterization of cardiac cell cultures at day 9. (A) Immunocytological double-staining for Cx43, α-SMA (MFB) or α-actinin (CMC). White arrows mark intercellular expression. (B) Western blot of primary CMC cultures, 50%/50% co-cultures of MFBs/CMCs, and purified MFB cultures. (C) Quantification of Western blots normalized for β-tubulin shows opposing trends of Cx43 and α-SMA expression related to MFB quantity. *:P<0.05 vs. CMC; **:P<0.05 vs. CMC and 50%/50%. (D) α-SMA and collagen-I double-staining in MFBs. (E) Collagen-I and α-actinin double-staining showing highly specificity for MFBs and CMCs, respectively. (F) Collagen-I and vimentin double-staining in MFBs. (G) Relationship between co-expression of α-SMA and collagen-I in MFBs, and (H) co-expression of vimentin and collagen-I in MFBs. FOV=Field of View.*

Uninhibited MFB proliferation and spontaneous reentrant arrhythmias

MFB percentage in primary cardiac cultures was 15.6±3.2% at day 1 and progressively increased to 37.4±1.7% at day 4 (P<0.0001) (Figure 2A). At day 2, a spontaneously beating confluent monolayer had formed. At day 4, 24.2% of these cultures showed sustained, spontaneous reentrant tachyarrhythmias (n=33) (Figure 2F), with an average cycle-length of 267±22 ms and conduction velocity (CV) of 15.3±3.5 cm/s. Sustained reentry was defined as repetitive circular activation lasting ≥30 s. MFB proliferation resulted in an MFB percentage of 62.2±2.0% at day 9 (P<0.0001 vs. day 1 and 4) (Figure 2B), and a decrease in CV to 8.8±0.3 cm/s (P<0.0001) (Figure 2C). At day 9, 81.3% of all spontaneously active

cultures showed reentrant activity (n=75) (Figure 2D). The cycle-lengths of these arrhythmias had increased to 365±57 ms (*P*<0.001 vs. day 4) (Figure 2E).

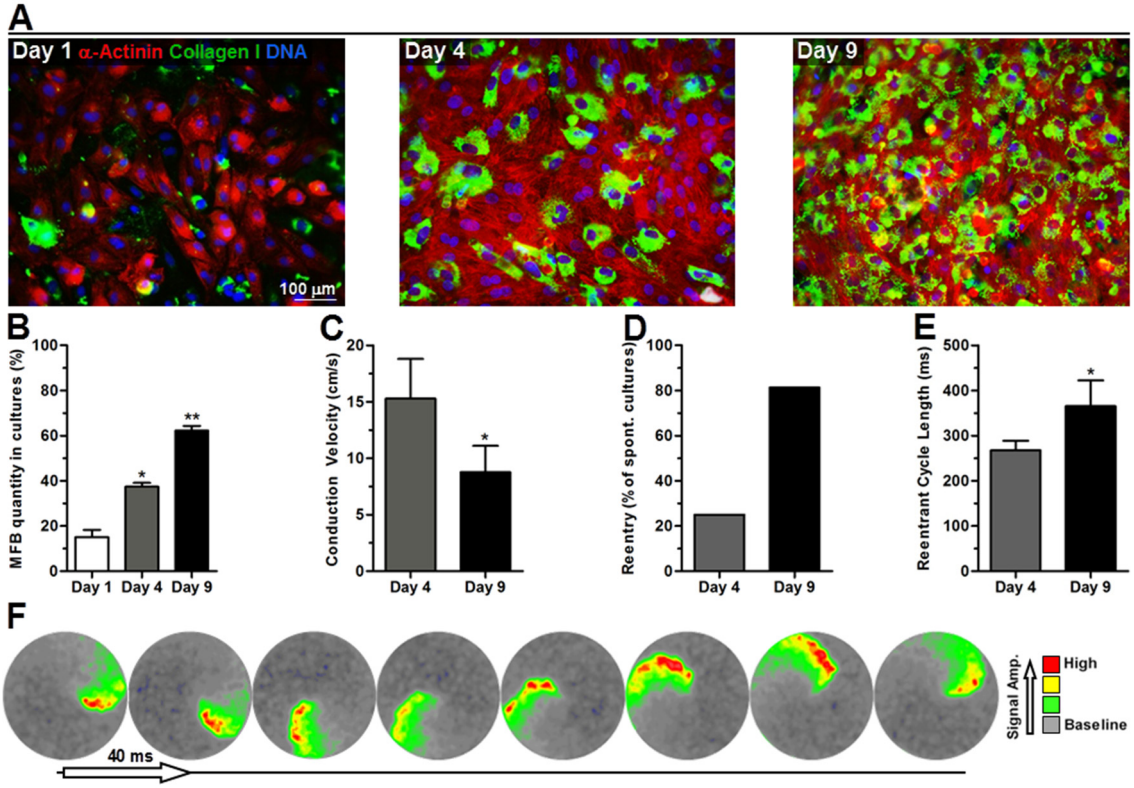


Figure 2. *Proliferation of MFBs in cardiac cultures causes conduction abnormalities. (A) Immunocytological staining for α-actinin (CMC;red) and collagen-I (MFB;green) in cardiac cultures at day 1, 4, and 9. (B) Quantification of collagen-I positive MFBs at day 1, 4 and 9. Quantities are expressed as a percentage of total number of nuclei. *:P<0.0001 vs. day 1. **:P<0.0001 vs. day 1 and 4. (C) Progressive increase in MFBs is associated with a lower CV. *:P<0.0001 vs. day 4, and (D) is also associated with an increase in the occurrence of spontaneous reentrant tachyarrhythmias and (E) an increase in cycle-length of the reentrant circuits between day 4 and 9. *:P<0.0001 vs. day 4. (F) Time-lapse (spacing: 40ms) of a typical high-pass-filtered, spatially averaged optical signal of reentrant activation. Colours represent signal intensities related to changes in membrane potential.*

Inhibition of MFB-proliferation preserves high CV, decreases ectopic activity and prevents reentrant arrhythmias

Assessment of proliferative activity of MFBs was performed by Ki67 staining (Figure 3A). Quantification showed a significant decrease in proliferating MFBs following mitomycin-C treatment (Figure 3B). In such cultures, MFB quantities remained constant throughout

follow-up (Figure 3C), with no significant increase in apoptosis compared to control (Figure 3D and Supplemental Figure 2).

Under mapping conditions, ≥60% of both treated and untreated cultures were spontaneously active at day 4 and 9. At day 4, CV of mitomycin-C treated cultures was 23±1.9 cm/s, which was significantly higher than in control cultures (~15 cm/s, *P*<0.0001) (Figure 4E). Furthermore, no arrhythmias were observed (n=17) in mitomycin-C treated cultures (Figure 4C, H). At day 9, CV in mitomycin-C treated cultures remained unaltered. Interestingly, at day 9, only 2.6% of spontaneously active mitomycin-C treated cultures showed sustained reentrant tachyarrhythmias (n=76), which is a dramatic decrease compared to proliferating control cultures (~81% arrhythmias (n=75) at day 9, Figure 4B, F-G and Movie 1).

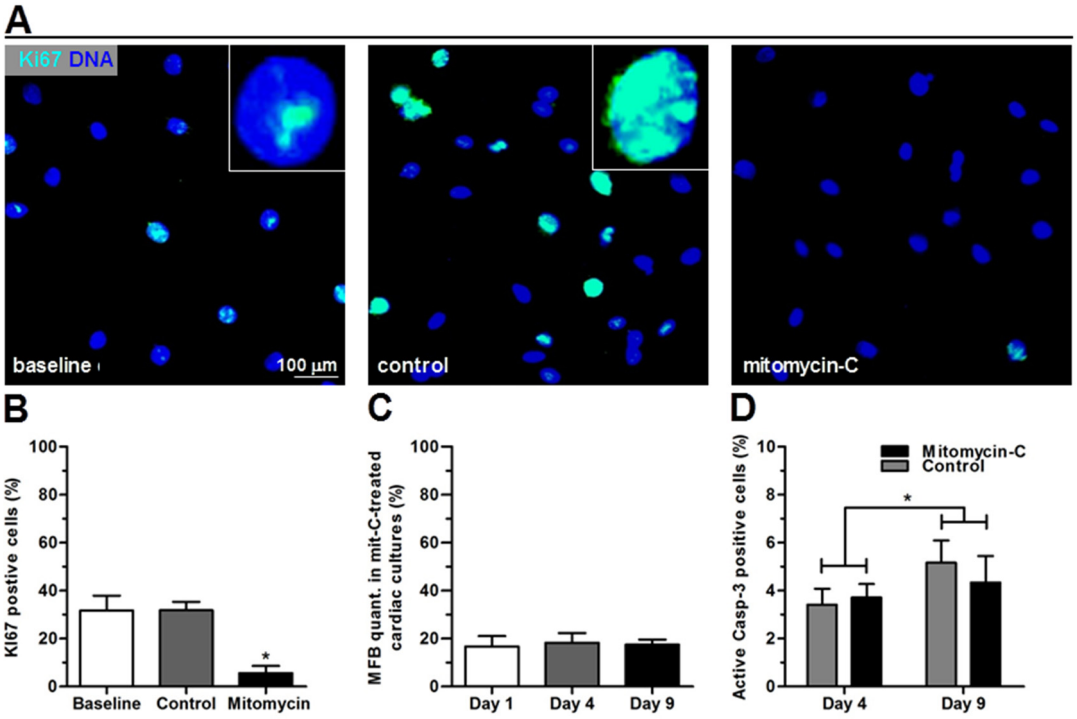
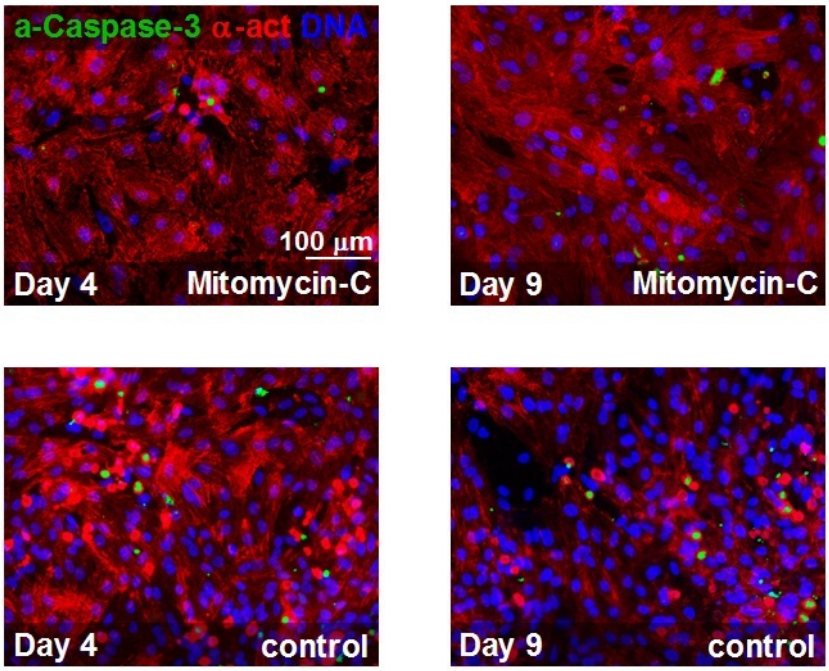


Figure 3. *Mitomycin-C is a potent inhibitor of MFB proliferation (A) Typical examples of Ki67 staining in MFBs, indicating proliferation at baseline (day 1) and 3 days later for control and mitomycin-C treated cultures. Insets show magnified nucleus positive for Ki67 staining. (B) Effect of mitomycin-C treatment on Ki67 positive staining in MFBs. *:P<0.001 vs. baseline and control. (C) MFB quantification in mitomycin-C treated cardiac cultures shows a stable MFB quantity throughout time (p=ns). (D) Quantification of active caspase-3 staining shows no significant differences in apoptosis between mitomycin-C treated and control cultures, although small but significant increases were found over time (*:P<0.05).*



Supplemental Figure 2. *Mitomycin-C does not increase apoptosis in myocardial cultures. Representative images of immunocytochemical staining for caspase-3 in mitomycin-C treated cultures and control cultures at day 4 and 9. Nuclei were counterstained with Hoechst and caspase-3 positive nuclei were quantified and expressed as a percentage of total cell count, which was not significantly affected by mitomycin-C treatment. Total cell count was defined as the total number of viable nuclei added to the amount of caspase-3 positive cells.*

For further evaluation of arrhythmogeneity, the incidence of ectopic activity was studied in treated and untreated cultures. Ectopic activity, e.g. multiple simultaneous or alternating pacemaker sites in one culture, was observed less frequently in mitomycin-treated cultures than in untreated cultures at day 4 (25% (n=24) versus 43% (n=23)) and day 9 (8% (n=37) versus 71% (n=35), respectively (Figure 4A, D). An example of how ectopic activity can lead to reentrant arrhythmias is demonstrated in Movie 3.

Analyses of extracellular electrograms from MEA mapping experiments showed distinct differences between control cultures (n=12) and mitomycin-C treated cultures (n=11) (Figure 4I-J). Peak-to-peak electrogram amplitude was higher in mitomycin-C treated cultures (702±304 μV vs. 96±23 μV, $P<0.0001$) (Figure 4K). Spontaneous electrical activation frequency was lowered by mitomycin-C treatment compared to control (0.28±0.22HZ vs.. 3.22±0.22Hz, $P<0.0001$) (Figure 4L).

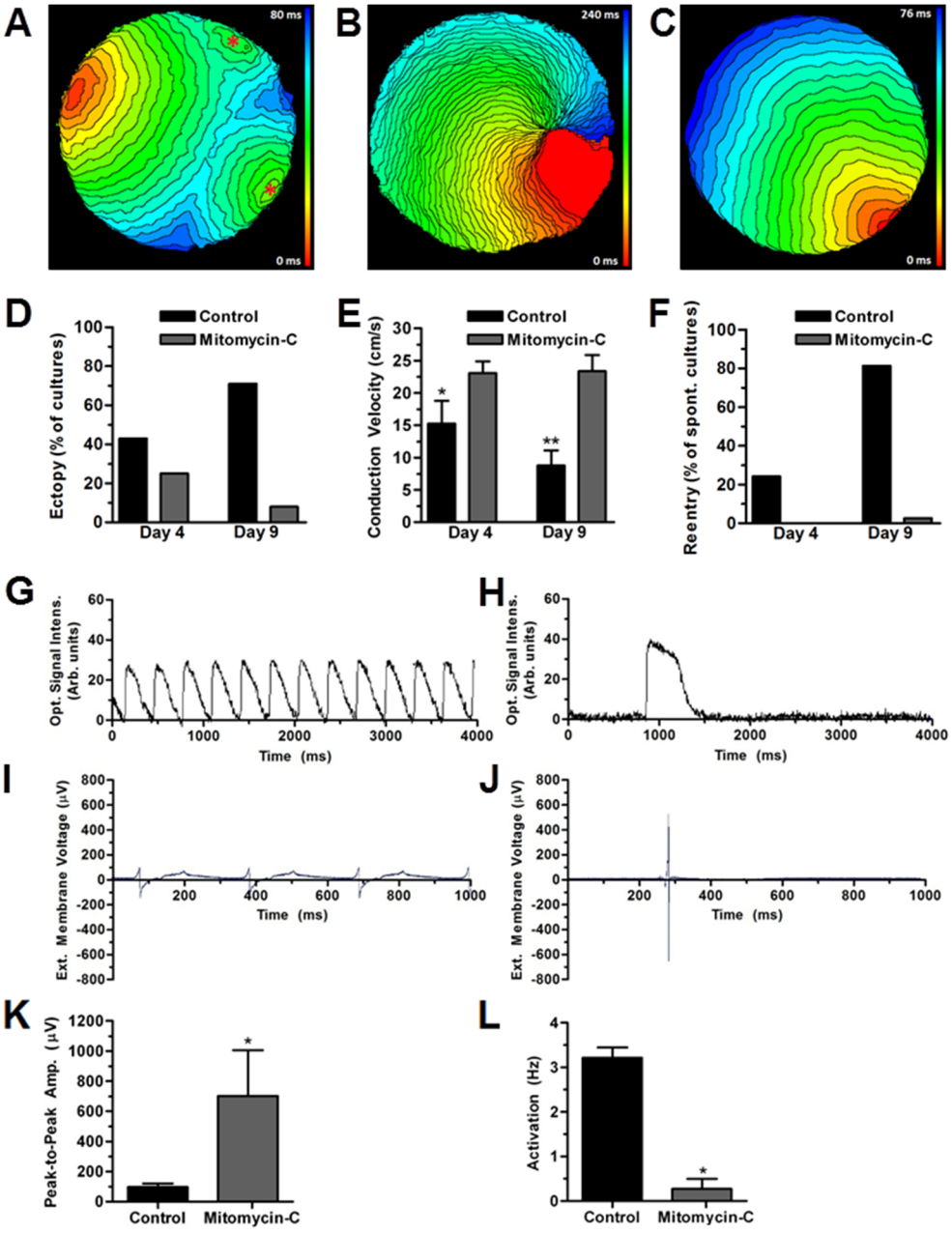


Figure 4. *Effects of mitomycin-C treatment on ectopic activity, CV and reentrant tachyarrhythmias in cardiac cultures. (A) Activation map of ectopic activity in an untreated culture at day 4 (4ms isochronal spacing). Red asterisks mark ectopic foci. (B) Activation map of a reentrant tachyarrhythmia in an untreated cardiac culture (spacing: 4ms). (C) Typical activation map of*

*uniform conduction across a mitomycin-C treated culture (spacing: 4ms). (D) Quantification of incidence of ectopic activity at day 4 and 9 reveals a substantial reduction by mitomycin-C treatment. (E) CV measured by optical mapping. *:P<0.001 vs. mitomycin-C day 4-9 and control day 9. **:P<0.001 vs. day 4-9 mitomycin-C. (F) Spontaneous reentry occurrence in mitomycin-C treated and control cultures at day 4 and 9. (G) Typical example of a non-high-pass-filtered, spatially filtered optical signal of repetitive activation in a non-treated, fibrotic culture showing reentrant tachyarrhythmias. (H) Typical example of a non-high-pass-filtered, spatially filtered optical signal of uniform conduction across a mitomycin-C treated culture. (I) Local extracellular multi-electrode array recording of a reentrant tachyarrhythmia. (J) Multi-electrode array recording of a mitomycin-C treated culture. (K) Quantification of electrical signal amplitude from multi-electrode array recordings at day 9 (P<0.0001 vs. control). (L) Beating frequency of cultures measured by such arrays at day 9. *: P<0.0001 vs. control.*

Dose-dependent effects of mitomycin-C treatment on preservation of electrophysiological parameters

As mitomycin-C treatment had such a profound impact on conduction properties of myocardial cultures, dose-dependency was studied next. Dosages administrated at day 1 of culture were 10, 5, 2.5, 0.5 and 0.05 μg/ml. At day 9, cultures were studied and subsequently stained for collagen-I (Figure 5A). Mitomycin-C decreased the amount of MFBs in a dose-dependent manner (Figure 5B). Furthermore, mitomycin-C had a strong dose-dependent effect on cell density (P<0.001). In addition, cardiomyocyte count, calculated by subtracting collagen-I positive cells from total cell count, did not change significantly (Figure 5C).

These dose-dependent changes in MFB quantities and cell density were related to significant electrophysiological changes in the cultures. At day 9, CV was 7.3±2.4 cm/s at 0.05 μg/ml mitomycin-C and significantly rose with increasing dosages (Figure 5D). MFB percentages at various mitomycin-C dosages directly correlated with CV (R^2=0.94) (Figure 5E). Furthermore, the incidence of sustained reentrant arrhythmias showed a negative mitomycin-C dose-dependent relationship, with no occurrence of arrhythmias at 10 μg/ml (n=25) and 5 μg/ml (n=20), 10% at 2.5 μg/ml (n=31), 29% at 0.5 μg/ml (n=17), 92% at 0.05 μg/ml (n=13), and 93% for control (n=27) (Figure 5F).

CMC-MFB co-cultures at predetermined cell density and MFB-dependent conduction abnormalities

Cell density is an important determinant of conduction patterns, as this directly influences cell-to-cell contacts essential for action potential propagation. To further study the quantitative effects of MFBs on conduction and arrhythmias, fixed ratios of MFBs and CMCs were plated out, while inhibiting proliferation with 10 μg/ml mitomycin-C. As a result, average cell density between the co-culture groups did not differ significantly at day 9.

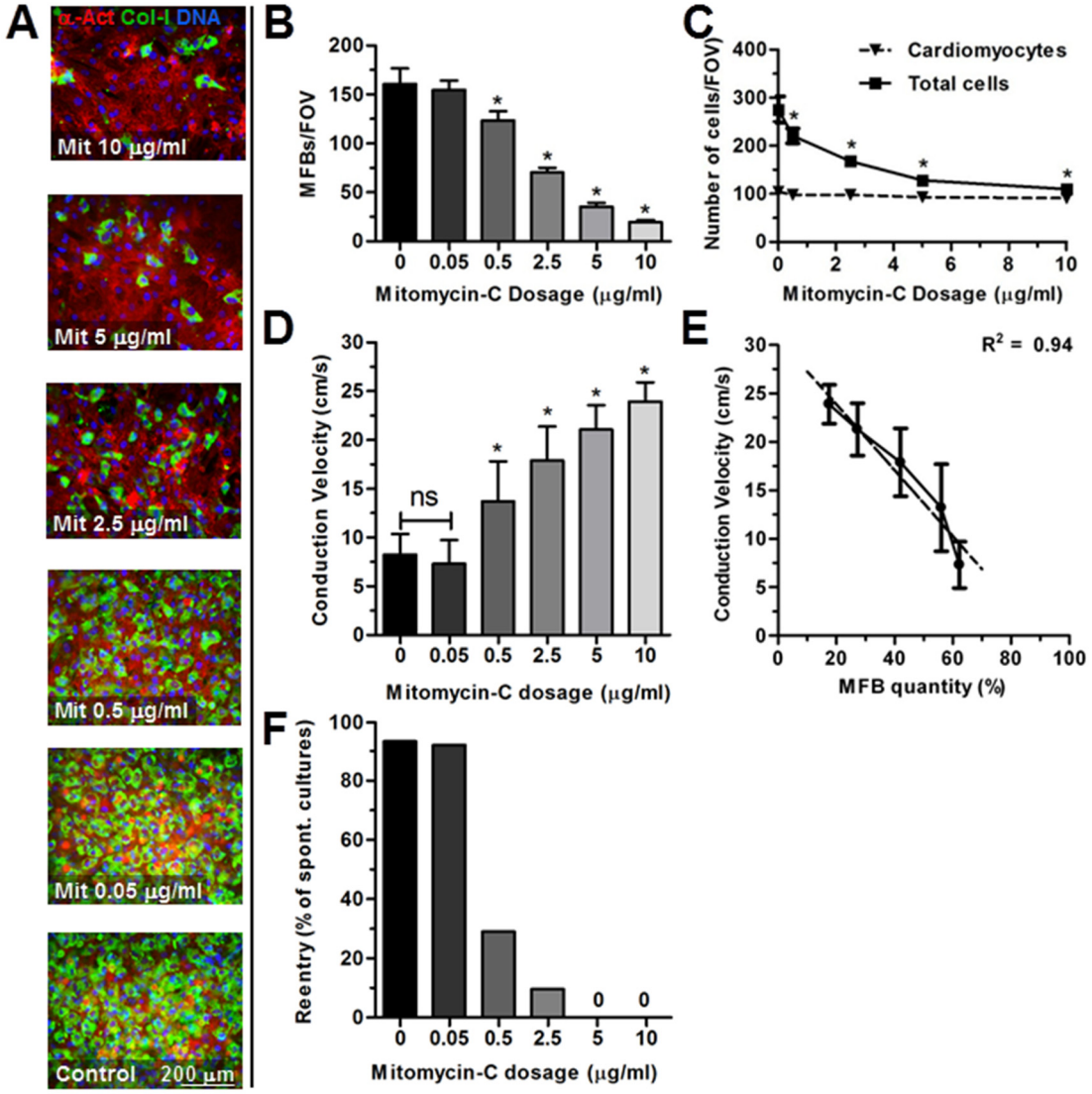
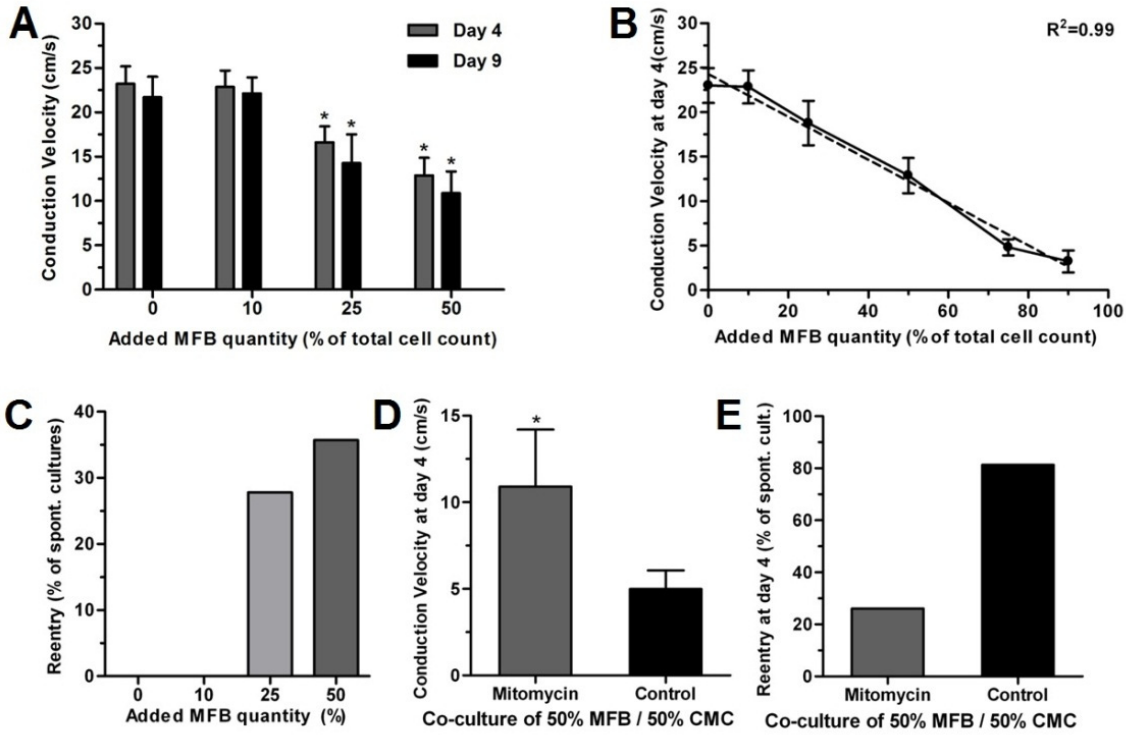


Figure 5. *Dose-dependent effect of mitomycin-C treatment on arrhythmias at day 9 of culture. (A) Immunocytological double-staining for α-actinin (CMC;red) and collagen-I (MFB;green) in cardiac cultures treated with different dosages of mitomycin-C. (B) Dose-dependent effect of mitomycin-C on MFB quantities in cardiac cultures as determined by collagen-I staining. *:P<0.001 vs. all including control (0 µg/ml). (C) Quantification of collagen-I staining shows a dose-dependent effect of mitomycin-C on total cell count without affecting CMC count. CMC count was calculated by subtract the number of MFBs from the total number of nuclei. *:P<0.01 vs. all including control. (D) Dose-dependent effect of mitomycin-C on CV (*:P<0.05 vs. all). (E) Plot of average MFB percentage found for different dosages against CV found in these groups shows a negative linear association between MFB percentage and CV. FOV: Field of View. (F) Dose-dependent effect of mitomycin-C on reentry occurrence.*



Supplemental Figure 3. *MFB-CMC co-cultures at predetermined cell density and the effects on conduction abnormalities. (A) CV slows according to plated MFB percentage, except for 10% MFBs. *:p<0.05 vs all. (B) CV at day 4 shows a strong linear association with added MFB percentage. Dotted line represents regression line. (C) Occurrence of spontaneous arrhythmias at day 9 is MFB quantity-dependent and rises with increasing added MFB percentages. (D) Mitomycin-C administration to 50%/50% MFB-CMC co-cultures results in a higher CV at day 4 compared to control. *:p<0.0001 vs control. (E) Uninhibited proliferation of MFBs in a 50%/50% CMC/MFB co-culture results in a 3.1-fold increase in reentry occurrence (n=23) compared to mitomycin-C treated cultures (n=17) of initially identical cellular composition.*

MFB quantities were 15.7±2.0% (0% added MFBs), 26.8±2.5% (10% added), 38.0±3 (25% added) and 59.0±1.6% (50% added) at day 9. CV did not differ significantly between 0% (n=18) and 10% (n=20) added MFBs (23.1±2.2 cm/s vs. 22.1±1.8 cm/s, p=0.51) at day 9. However, 25% and 50% added MFBs slowed conduction to 14.2±3.5 cm/s and 10.9±2.4 cm/s, respectively (*P*<0.05 vs.. all) (Supplemental Figure 3A). Furthermore, no arrhythmias were found in cultures containing 0% and 10% added MFBs, but at 25% and 50% added MFBs, occurrence was 27.8% (n=36) and 35.7% (n=28), respectively (Supplemental Figure 3C).

Unfortunately, cultures with added MFB percentages higher than 50% developed structural inhomogeneities from day 6 onwards and could therefore not be studied at day 9. Nevertheless, linear regression analysis revealed a strong inverse relationship between plated MFB percentages and CV at day 4 (Supplemental Figure 3B).

A potentially secondary preventive effect of mitomycin-C on the occurrence of spontaneous arrhythmias was studied in 50%/50% CMC/MFB co-cultures either treated with mitomycin-C or allowed to proliferate freely. At day 4, CV in mitomycin-C treated cultures was 10.9±3.3 cm/s with 60.2±3.8% MFBs. In contrast, in control cultures with an equally high initial number of MFBs, CV decreased to 4.9±1.1 cm/s, while MFB percentages increased to 78.8±4.7% (P<0.0001 vs. treated cultures) (Supplemental Figure 3D). Furthermore, arrhythmia occurrence was 3.1-fold higher in the non-treated cultures (26% (n=17) vs. 82% (n=23)) (Supplemental Figure 3E).

Characteristics of reentrant tachyarrhythmias

In untreated, arrhythmic cultures, cycle-length of the reentrant circuits was strongly related to CV (R^2=0.83, Figure 6A). Reentry was typically associated with a decrease in CV of 5.0±1.2 cm/s as compared to non-reentrant conduction in cultures from the same experimental group. Furthermore, administration of tetrodotoxin (TTX) to 12 untreated, arrhythmic cultures at concentrations of 5 μM and 20 μM at day 9 of culture, resulted in a significantly lower CV (Figure 6B, D-E), but had only a mild to moderate effect on terminating reentrant arrhythmias (Figure 6C). Next, 100 μM verapamil was administered to block L-type Ca^{2+}-channels, which terminated 100% of the remaining arrhythmias (Figure 6C and Movie 2). Internal PBS control did not affect arrhythmia persistence. Additionally, 12 untreated, arrhythmic cultures were immediately treated with verapamil without prior TTX administration, which also terminated all arrhythmias. In mitomycin-C treated cultures, 20 μM TTX completely blocked propagation for ≥30 seconds, after which propagation resumed at a significantly lower CV of 11.5±2.0 cm/s (n=10, previously 24.2±2.0 cm/s, P<0.0001).

To further study the role of MFB proliferation in arrhythmogeneity, CMCs were investigated for their electrophysiological properties by patch-clamp experiments in active cultures treated with or without mitomycin-C. Cultures had comparable beating frequencies (0.5-1 Hz). After 9 days of on-going MFB proliferation, the maximal negative diastolic potential of CMCs was significantly reduced (-44±9mV, n=11) as compared to those of CMCs in mitomycin-C treated cultures (-68±7mV, n=12, P<0.001) (Figure 6F).

In co-cultures of eGFP-labelled MFBs with CMCs, at equal density and ratio as day 9 of free proliferation, diastolic membrane potentials of CMCs (-48±6mV, n=8) were comparable to those derived at day 9 of free proliferation. This is in agreement with the low CV and increased ectopic activity found in such cultures and their tolerance to TTX treatment.

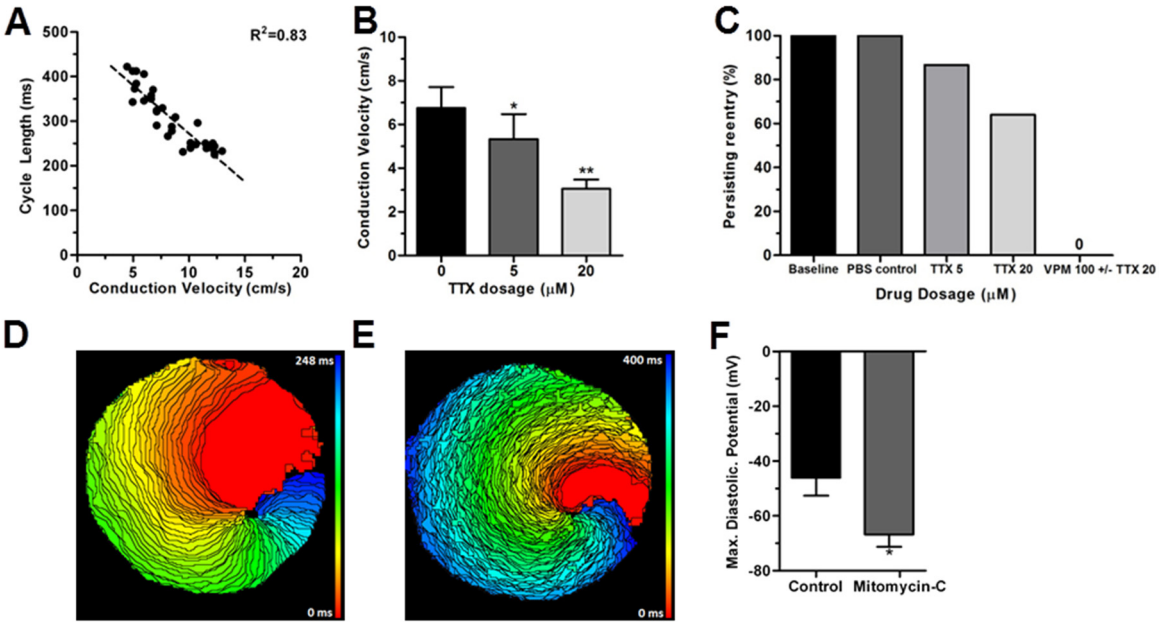
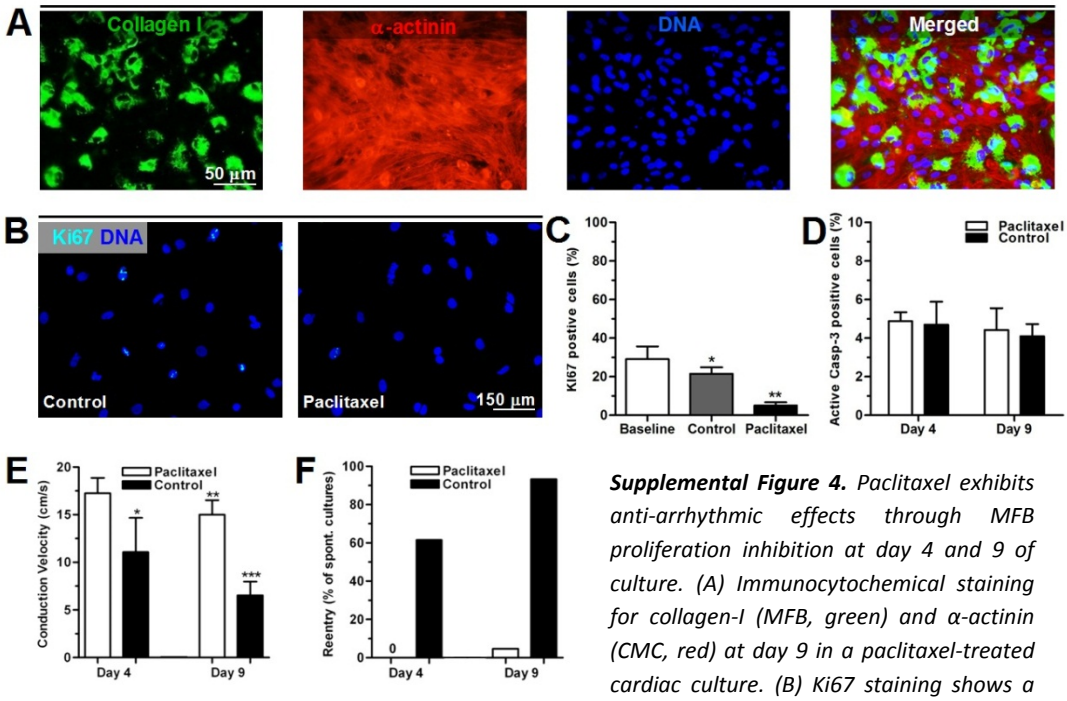


Figure 6. *Characteristics of reentrant tachyarrhythmias at day 9. (A) Linear association between cycle-length and CV (R^2=0.83, n=35). (B) TTX significantly decreased CV in a dose dependent manner (*:P<0.05 vs. baseline, **:P<0.0001 vs. 0 and 5 μM). (C) Persistence of reentrant circuits after administration of TTX and/or verapamil. (D) Activation map of a reentrant tachyarrhythmia in an untreated cardiac culture (spacing: 4ms). (E) Activation map of the same reentrant tachyarrhythmia shown in panel E after 5 min of incubation with 20 μM TTX. Of note is the increased number of isochronal lines (4 ms), indicating conduction slowing. (F) Maximal diastolic potential measured in CMCs from control and mitomycin-C treated cultures at day 9.*

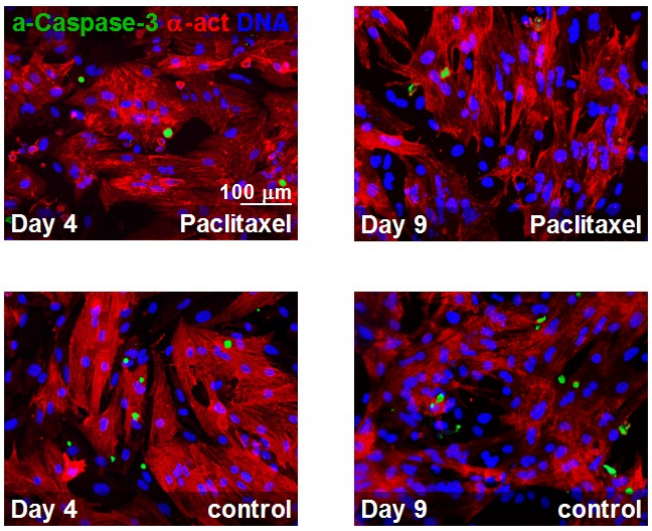
Prevention of arrhythmias by paclitaxel, another antiproliferative agent

Cultures were treated with 0.085 mg/ml paclitaxel and studied identically to mitomycin-C treated cultures. In paclitaxel-treated MFB cultures, less Ki67 positive cells were found (Supplemental Figure 4B-C) and apoptosis was not significantly increased compared to vehicle-control (0.9% DMSO) treated cultures (Supplemental Figure 4D, 5). Interestingly, vehicle treatment alone also inhibited proliferation. Spontaneous activity under optical mapping conditions was >60% for both treated and untreated groups. CV at day 4 was 17.3±1.6 (vs. 11.1±3.6 cm/s in control), without reentry (n=27) in the paclitaxel-treated group, whereas 61.5% (n=13) of controls showed reentry (Supplemental Figure 4E-F). At

day 9, paclitaxel-treated cultures contained 33.3±2.9% MFBs (Supplemental Figure 4A), and CV was 15.0±1.5 cm/s (*P*<0.01 vs. day 4 and control), while arrhythmias were observed in 5% of spontaneously active cultures (n=23). In control cultures at day 9, CV was 6.5±1.4 cm/s, with a reentry incidence of 93% (n=15) (Supplemental Figure 4E-F).



Supplemental Figure 4. *Paclitaxel exhibits anti-arrhythmic effects through MFB proliferation inhibition at day 4 and 9 of culture. (A) Immunocytochemical staining for collagen-I (MFB, green) and α-actinin (CMC, red) at day 9 in a paclitaxel-treated cardiac culture. (B) Ki67 staining shows a substantial reduction in positive cells by paclitaxel administration. (C) Quantification of Ki67-positive MFBs before and after paclitaxel treatment. *:p<0.05 vs baseline, **:p<0.05 vs baseline and control. (D) Quantification of apoptotic cells by active caspase-3 staining shows no significant increase in apoptosis by 10 μmol/L paclitaxel. (E) Paclitaxel-treated cultures maintain a higher CV compared to control cultures. *:p<0.05 vs paclitaxel at day 4. **:p<0.05 vs paclitaxel at day 4 and control at day 9. ***:p<0.05 vs control at day 4 and paclitaxel at day 9. (F) Paclitaxel treatment dramatically decreased reentry occurrence compared to control at both day 4 and 9 of culture.*



Supplemental Figure 5. *Representative images of active caspase-3 immunocytochemical staining in paclitaxel-treated cultures and control cultures at day 4 and 9. Nuclei were counterstained with Hoechst and active caspase-3 positive nuclei were quantified and expressed as a percentage of total cell count, which was not significantly affected by paclitaxel treatment. Total cell count was defined as the total number of viable nuclei added to the amount of caspase-3 positive cells.*

Discussion

Key findings of this study are (1) proliferation of myofibroblasts in myocardial cultures results in a highly pro-arrhythmogenic substrate, in which CMCs are depolarized, conduction is slow and mainly Ca^{2+}-driven, and ectopic activity is increased, thereby giving rise to spontaneous, sustained reentrant tachyarrhythmias, and (2) antiproliferative treatment of these cultures prevents or substantially reduces the occurrence of arrhythmias by limiting myofibroblasts-induced depolarization and preserving uniform, rapid, Na^{+}-driven impulse propagation in CMCs, with less ectopic activity, but without noticeable adverse effects on electrophysiological properties and without increased apoptosis in the treated cultures.

Myofibroblasts and Cardiac Arrhythmogeneity

In vitro studies indicate that MFBs could play a role in modulating electrophysiological properties in remodeled hearts, and thereby contribute to arrhythmogenesis. Rook *et al*. showed that cardiac fibroblasts and CMCs are able to form functional heterocellular gap junctions.[9] Gaudesius *et al*.,[10] and previous studies by our group,[11,12] demonstrate that fibroblasts coupled to CMCs are able to slowly conduct electrical impulses through electrotonic interaction. Paracrine activity of cardiac fibroblasts may also contribute to a reduction in CV.[13] Besides their effects on CV, MFBs may also induce ectopic activity in cardiac cultures as demonstrated by Miragoli *et al*.[14] Recently, a study by Zlochiver *et al*. showed that MFBs are able to contribute to rhythm disturbances in cardiac cultures,[15] which was further investigated in a number of *in silico* studies.[16,17] Novel in the present study is the finding that ongoing proliferation of endogenous MFBs in neonatal rat CMC

cultures results in the creation of a highly arrhythmogenic substrate, and that antiproliferative treatment of cardiac cultures prevents spontaneous reentrant tachyarrhythmias.

Reentrant tachyarrhythmias in cardiac cultures with ongoing MFB proliferation

In previous studies, functional reentry was induced by rapid electrical pacing of the cultures.[15,18,19] In the present study the focus was on the ability of myocardial cultures to spontaneously generate such reentrant arrhythmias. MFBs are known to contribute to both automaticity and slow conduction,[10,14,20] therefore reentry may occur in the absence of externally applied electrical impulses. We confirmed this by showing that ~81% of all cultures with ongoing MFB proliferation and without apparent anatomical obstacles, showed spontaneous, sustained reentrant tachyarrhythmias at day 9 of culture.

By allowing MFBs to proliferate freely in CMC cultures, CMCs became increasingly depolarized due to increasing MFB-CMC interactions. The present study shows that this eventually leads towards a depolarized resting membrane potential at which voltage-gated fast Na^+-channels are largely inactivated and propagation becomes mainly dependent on activation of Ca^{2+}-channels. It is known that Ca^{2+}-driven propagation contributes to slow conduction.[7] In line with this observation, all arrhythmias in the present study were terminated when L-type Ca^{2+}-channels were blocked. In contrast, most arrhythmias sustained after Na^+-channel blockade, indicating that electrical propagation in such conditions appears to be mainly Ca^{2+}-driven. Recently, Chang *et al*. showed similar results in another *in vitro* model of reentry, using non-fibroblastic cells.[19] Moreover, as these CMCs become depolarized by increasing numbers of MFB, they could become active as local pacemaker site through depolarized-induced automaticity,[21] and thereby add to the pro-arrhythmogenic nature of fibrotic cultures. The present study shows that around 50% of all fibrotic cultures, which did not show reentrant arrhythmias at the time of mapping, showed multiple simultaneous or alternating pacemaker sites.

Ongoing MFB proliferation also had an effect on the cycle-length of the tachyarrhythmias. At day 4, average cycle-length was 267±22 ms at a CV of 15.3±3.5 cm/s, whereas at day 9, the cycle-length increased to 365±57 ms at a CV of only 8.8±3 cm/s. These data may explain how different degrees of fibrosis during various stages of cardiac remodelling could both determine the vulnerability to arrhythmias and the rate of atrial or ventricular arrhythmias. Therefore, future *in vivo* studies are required to better understand the role of MFBs in the arrhythmic heart.

Antiproliferative treatment of endogenous myofibroblasts

In the present study, two different antiproliferative agents were used to study the role of MFB proliferation in arrhythmogeneity. One of these agents is mitomycin-C, a potent DNA-crosslinking agent. After proliferation inhibition by mitomycin-C, CV remained stable

from day 4 to day 9, and spontaneous reentry occurrence decreased from 81.3% to 2.6%. Concerning the underlying mechanisms, in cultures treated with mitomycin-C the resting membrane potential of CMCs remained more negative. Therefore propagation remained fast and mainly Na^+-driven, as was shown by addition of TTX to these cultures, in contrast to cultures with ongoing MFB proliferation. In addition, less ectopic activity was observed in cultures treated with mitomycin-C, which most likely resulted from limited MFB-induced depolarization and a subsequent reduction of the occurrence of depolarization-induced automaticity. As a consequence of fast propagation and less ectopic activity the occurrence of reentrant arrhythmias is expected to decrease, which was confirmed in the present study.

By inhibition of MFB proliferation, not only MFB-induced depolarization of CMCs is minimized, but also disruption of low-resistant gap junctional coupling between CMCs by infiltrating MFBs may be prevented, thereby preserving rapid propagation. Calculations on cell densities indicated that antiproliferative treatment does coincide with a lower total cell density while maintaining the same number of CMCs as the non-treated cultures. In addition, any negative, paracrine effect of MFBs on CV in cardiac cultures will be stabilized after inhibition of proliferation; as such an effect is expected to be cell number-dependent.

To exclude a mitomycin-C specific effect and to establish that proliferation is the key factor in this study, another antiproliferative agent was studied. Paclitaxel, a member of the taxanes drug category, interferes with breakdown of microtubules during cell division. Park *et al*. used the antiproliferative potential of paclitaxel to inhibit coronary restenosis and neo-intimal hyperplasia in the myocardium.[22] We show that paclitaxel is also suited as agent to reduce the incidence of reentrant tachyarrhythmias in myocardial cultures. Control experiments for paclitaxel included incubation with DMSO (0.9%). DMSO is known to have several effects on cells,[23] and therefore may explain the high incidence of reentry at day 4 in these cultures. The lower CV found in paclitaxel-treated cultures may also be explained by its mechanism of action.[24] Nevertheless, no reentry was observed in paclitaxel-treated cultures with DMSO as vehicle. Importantly, both agents did not result in increased apoptosis, which is in agreement with earlier studies.[25,26]

In vivo translation

The present study provides new insights in the way cardiac fibrosis may result in arrhythmias, and how this may provide a rationale for a preventive strategy, which currently does not exist. In the clinical setting, myocardial fibrosis increases arrhythmia vulnerability in diseased and aged hearts, and finds its basis in proliferation of MFBs and matrix deposition by these cells. Although functional MFB-CMC coupling remains to be proven *in vivo*, the key role of MFBs in cardiac fibrosis suggests a high significance for *in vivo* arrhythmogeneity of these cells. Measures to control MFB proliferation may

therefore counteract different pro-arrhythmic aspects at once. Naturally, *in vivo* studies are necessary to determine whether progressive fibrosis (e.g. in post myocardial infarction or aging) and its pro-arrhythmic consequences can be limited by reducing MFB proliferation. Considering the role of cell proliferation in different physiological processes in the heart,[5,27,28] and possible cardiotoxic effects of antiproliferative agents,[29] careful consideration of the time-frame, location and strength of intervention seems of importance. Still, the strong *in vitro* evidence from this study suggests that approaches to limit MFB proliferation in hearts vulnerable to fibrosis-related conduction disturbances may have profound effects on arrhythmia vulnerability.

Study limitations

The use of adult human CMCs and MFBs may have been more clinically relevant, but these CMCs cannot be kept in culture for longer periods and the proliferation rate of such MFBs *in vitro* does not allow a study like this within a reasonable time-frame. Furthermore, cardiac MFBs are also involved in secretion of extracellular matrix components, which could contribute to deleterious effects on conduction in fibrotic cardiac tissue. These aspects were not studied in detail and need more dedicated studies in the future. However, it may be expected that with inhibition of MFB proliferation, the secretion of such components is indirectly lowered.

Conclusions

Proliferation of MFBs in myocardial cultures gives rise to spontaneous, sustained reentrant tachyarrhythmias. However, antiproliferative treatment of such cultures prevents the occurrence of arrhythmias significantly by preserving a physiological membrane potential and rapid, Na^+-driven propagation in CMCs. Hence, our study indicates that MFB proliferation may be a novel target for future anti-arrhythmic strategies.

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Conflict of Interest

None.

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.

Cell isolation and culture

Primary neonatal rat cardiomyocytes (CMCs) and cardiac fibroblasts were isolated and cultured as described previously[30,31]. Immediately following isolation, cell suspensions were spinned down, resuspended and filtered through a 20 μm cell-strainer to remove cell-aggregates. Subsequently, cells were counted and plated out on fibronectin-coated round coverslips (15 mm) at a cell density of 4-8x10^5 cells/well in 24-well plates (Corning Life Sciences, Amsterdam, the Netherlands), depending on the experiment.

For co-culture experiments with fixed myofibroblast (MFB) quantities (0%, 10%, 25%, 50%, 75%, and 90%), primary CMCs, and MFBs obtained from an earlier isolation (passage 3-4), were counted and mixed in specific, pre-determined ratios before plating. Co-cultures were treated with mitomycin-C (Sigma-Aldrich, St. Louis, MO, USA) to maintain original CMC-fibroblast ratios. All cultures were refreshed daily and cultured in a humidified incubator at 37ºC and 5% CO_2.

Antiproliferative treatment

Antiproliferative treatment of cultures was performed at day 1 of culture. The choice of different types of antiproliferative agents was based on their specific mechanisms of action, thereby allowing a more accurate study of the role of proliferation in arrhythmogeneity, rather than an agent-specific effect.

Different dosages of the antiproliferative agent of interest (mitomycin-C dissolved in PBS; ranging from 10 μg/ml to 0.05 μg/ml or paclitaxel dissolved in DMSO; 0.085 mg/ml, both from Sigma-Aldrich) were diluted in growth medium (Ham's-F10 supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 10% horse serum (HS, Invitrogen), and penicillin (100 U/ml) and streptomycin (100 μg/ml, P/S; Bio-Whittaker, Carlsbad, CA, USA)) and incubated for 2 hours. Cultures were then rinsed twice in PBS and once in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and Ham's F10 medium (ICN Biomedicals, Irvine, CA, USA) supplemented with 5% HS and P/S, before being kept on this medium throughout the experiment. Non-treated controls were rinsed identically.

Immunocytochemical analyses

Following mapping experiments, cultures were stained for proteins of interest, or cultures were stained parallel to mapping experiments, as described in earlier studies[30]. Cultures were stained for α-smooth muscle actin (α-SMA) and vimentin expression to study fibroblasts phenotype (Sigma-Aldrich), collagen-I expression to quantify myofibroblast (MFB) numbers (Abcam, Cambridge, MA, USA), Ki67 expression to identify actively proliferating cells (Abcam) and α-actinin as cardiomyocyte-specific marker (Sigma-Aldrich). Furthermore, cultures were also stained for connexin 43 (Cx43) (Sigma-Aldrich) to study gap junction formation between CMCs and MFBs. Alexa donkey-anti-mouse IgG 568 and Alexa donkey-anti-rabbit IgG 488 secondary (Invitrogen) antibodies were used at a dilution of 1:400. For all staining, nuclei were counterstained using Hoechst 3342 (Invitrogen). A fluorescent microscope equipped with a digital camera (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 the 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 taken at different magnifications (10, 40, 100x). All cultures were stained using the same solutions and captured using equal exposure times for the protein of interest.

Western-blot analyses

Cx43 expression was studied in a MFB-density dependent manner and correlated with α-SMA expression. Homogenates were made from 3 different purified CMC cultures, 50%/50% CMC/MFB co-cultures and purified MFB cultures, size-fractionated on NuPage 12% Tris-Acetate NuPage gels (Invitrogen) and transferred to Hybond PVDF membranes (GE Healthcare, Waukesha, WI, USA). These membranes were incubated with antibodies against Cx43 or α-SMA (both from Sigma-Aldrich) for 1 h followed by incubation with corresponding HRP-conjugated secondary antibodies (Santa Cruz Biotechnologies,Santa Cruz, CA, USA). β-tubulin (Millipore, Billerica, MA, USA) expression was determined to check for equal protein loading. Chemiluminescence was induced by ECL advance detection reagents (GE Healthcare) and caught on Hyperfilm ECL (GE Healthcare), after which the intensity of Cx43 and α-SMA bands were quantified by Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

Proliferation assays

To assess the effect of antiproliferative treatments on MFB proliferation, MFB cultures were seeded at $5x10^4$ cells/well in a 24-wells plate and stained for Ki67 (Abcam). Ki67 is a cellular marker for cell proliferation, and was quantified for positive staining in MFBs, before and 3 days after treatment. Quantification was performed on 6 cultures from

which 8 images were taken per culture at 40x magnification. These cultures were kept on DMEM/Ham's F10 + 5% HS and refreshed daily. Furthermore, collagen-I staining was performed after optical mapping experiments on at least 6 cardiac cultures to determine the number of MFBs in cultures treated with different antiproliferative agents and dosages (mitomycin-C 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 0.5 µg/ml, 0.05 µg/ml) at day 1, 4 and 9. Quantification of total nuclei count, and cells expressing collagen-I was performed on at least 6 images and averaged per culture per timepoint per dosage.

Apoptosis assay

Antiproliferative treatment may lead to increased apoptosis. Therefore, we investigated the expression of active caspase-3 (Abcam), an established marker for apoptosis, in cultures treated with antiproliferative agents and appropriate controls. These cardiac cultures were seeded at $4x10^5$ cells/well in a 24-wells format. The same protocol was used as described earlier for immunocytochemical staining. Total nuclei number and cells expressing active caspase-3 were quantified within the same image and averaged for 6 images per culture for 6 different cultures at 40x magnification.

Optical and multi-electrode mapping

To investigate action potential propagation patterns on a whole-culture scale, cultures ($8x10^5$ cells/well in a 24-wells format), treated with or without antiproliferative agents, were optically mapped with the voltage-sensitive dye di-4-ANEPPS (Invitrogen), on day 4 and 9. For reasons of standardization and reproducibility, only spontaneously active cultures with high degrees of structural and functional homogeneity (determined by light-microscopy and electrophysiological mapping) were included for further analyses. As a result, 95 out of every 100 cultures (wells) were included. On days of mapping experiments, cells were incubated with culture medium (DMEM/Ham's F10 + 5% horse serum) containing 16 µmol/L di-4-ANEPPS for 15±5 minutes. Following incubation, cells were refreshed with DMEM/Ham's F12 (37ºC) and immediately mapped. Mapping experiments in a 24-well plate typically did not exceed 30 min. Excitation light (λ_{ex}=525±25nm) was delivered by a halogen arc-lamp (MHAB-150W, Moritex Corporation, San Jose, CA, USA). Fluorescent emission light (λ_{em}>590 nm) was passed through a camera lens (1x Plan-Apo, WD=15 mm; Leica, Wetzlar, Germany) and 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. During measurements, cultures were kept at 37ºC. Electrical activation was recorded for at least 4 seconds at a rate of 500 frames/s, high-pass filtered and analyzed using Brain Vision Analyze 0909 (Brainvision Inc, Tokyo, Japan). The same culture was never exposed for longer than 40 s to minimize phototoxic effects. Importantly, mapping of the same culture at both day 4 and 9 appeared to be well tolerated as no structural

inhomogeneities were observed and CV did not change significantly over time in mitomyocin-C treated cultures.

Each pixels' signal was averaged in a fixed grid of 3x3 pixels. Activation time points were determined at dF/dt_{max}, which corresponds to the timepoint of maximum upstroke velocity. Conduction velocity (CV) in cultures with a uniform activation pattern was calculated between two 3 by 3 pixel grids, typically spaced 2-8 mm apart, and perpendicular to the activation wavefront. For cultures showing reentrant conduction wavefronts, CV was determined similarly at half the maximal distance from the core perpendicular to the wavefront over a length of 2-4 mm. Per culture, CV was determined in 6-fold and averaged for further comparisons. Reentrant cycle length was calculated from 3 separate cycles per culture and averaged. As this study focuses on reentry occurrence and prevention of such arrhythmias, all CV values for reentry and non reentry cultures were pooled within each different group, as this inclusion makes CV a valuable parameter in determining therapeutic effects *in vitro*.

For multi-electrode array (MEA) mapping, cells were cultured in glow-discharged, fibronectin-coated MEA culture dishes (Multi Channel Systems, Reutlingen, Germany) and measurements were performed as described previously [31], typically within 10 seconds after optical mapping.

Assessment of ectopic activity

Spontaneous ectopic activity was defined as multiple simultaneous or alternating pacemaker sites. In our cultures, 53% of fibrotic cultures (n=47) versus 23% (n=44) in mitomycin-C treated cultures showed ectopic activity spontaneously at day 4. However, at day 9 a high percentage of non-treated cultures showed spontaneous reentry, which continuously excites the cardiac tissue and thereby greatly decreases the possibility of ectopic activity. Therefore, to be able to quantify ectopic activity at day 9, reentry needed to be eliminated in a non-pharmacological manner. For this purpose, we used a custom-made epoxy-coated platinum electrode and performed unipolar stimulation with 6 V for 4 seconds using an electrical stimulus module with corresponding software (Multichannel Systems), which successfully eliminated re-entry in >90% of the cultures. This allowed for spontaneous ectopic activity to resume, which was detected by mapping of the cultures for 24 seconds directly after applying the stimulus. This allowed for quantification of ectopic activity at day 9. To provide a balanced comparative view, these experiments were performed identically at day 4 and 9 in mitomycin-C treated and control cultures.

Whole-cell patch-clamp

Whole-cell patch-clamp measurements were performed in co-cultures of CMCs and MFBs treated with or without mitomycin-C at day 9 of culture. In addition, MFBs were labeled with eGFP using lenti-viral vectors (LV.CMV.eGFP.HBVPRE; MOI 10), refreshed daily and passaged twice before these cells were co-cultured with CMCs at equal density and ratio at day 9 of free MFB proliferation. After identification of CMCs by phase contrast or fluorescence microscopy, maximal diastolic potentials in CMCs were recorded in current-clamp. Whole-cell recordings were performed at 25°C using a L/M-PC patch-clamp amplifier (3kHz filtering) (List-Medical, Darmstadt, Germany). The pipette solution contained (in mmol/L) 10 Na_2ATP, 115 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES/KOH (pH 7.4). Tip and seal resistance were 2.0-2.5 MΩ and >1 GΩ, respectively. The bath solution contained (in mmol/L) 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH 7.4). For data acquisition and analysis, pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) was used.

Dye transfer

To establish functional coupling between MFBs and CMCs, MFBs were labeled with a lenti-viral vector encoding kathuska (MOI 20). MFBs were kept in culture for 2 weeks and were at least passaged twice before usage in experiments. CMC cultures with a density of $1.0x10^5$ 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 30 minutes with calcein-AM (Invitrogen) diluted in HBSS (Gibco), which once internalized is converted to the green fluorescent dye calcein. Cells were rinsed twice with PBS and kept on culture medium containing 2.5 mmol/L probenecid (Invitrogen) to block extracellular leakage. Kathuska labeled MFBs were subsequently plated out in a 1:1 ratio with the calcein-loaded CMCs. Brightfield and fluorescent images (at least 10 per culture) were captured after 24 hours.

Pharmacological interventions

To study the role of Nav1.5 and Cav1.2 channels in the maintenance of reentrant arrhythmias in cultures with high numbers of MFBs, increasing doses of Tetrodotoxin (TTX) (5 and 20 μmol/L; Sigma-Aldrich) were added to the cultures followed by administration of verapamil (100 μmol/L; Centrafarm, Etten-leur, the Netherlands) during optical mapping experiments. As internal control, PBS was administered to the mapping medium prior to pharmacological interventions. To study the role of Cav1.2 blockade exclusively, arrhythmic cultures were immediately treated with verapamil without prior TTX administration. Both 20 μM TTX and 100 μM verapamil are expected to fully block the targeted ion channels in neonatal rat CMCs[32,33]. The electrophysiological effects of these drugs were evaluated by optical mapping, directly, 1 min and 5 min after administration.

In identical fashion, TTX was administered to mitomyocin-C treated cultures at day 9, showing uniform, rapid activation, and evaluated for persistence of electrical activation.

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 if appropriate, and expressed as mean±SD. Linear correlation analysis was performed by calculating Pearson's correlation coefficient. Comparison between two groups was performed using the student-t test. Differences were considered statistically significant if $p<0.05$.

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