Antiproliferative treatment of myofibroblasts prevents arrhythmias in vitro by limiting myofibroblast-induced depolarization

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Aims
Cardiac fibrosis is associated with increased incidence of cardiac arrhythmias, but the underlying proarrhythmic 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 and results
Endogenous MFBs in neonatal rat CMC cultures proliferated freely or under control of different dosages of antiproliferative agents (mitomycin-C and paclitaxel). At Days 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), whereas 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 Ca2+-channel blockade terminated 100% of reentrant arrhythmias, in contrast to Na+ blockade (36%, n = 12).

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.

Keywords
Myofibroblasts • Proliferation • Arrhythmia • Mapping • Prevention

1. Introduction
Cardiac arrhythmias remain a leading cause of mortality in the Western world, despite a variety of treatment options. 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. 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. Furthermore, antiarrhythmic 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. The limited therapeutic efficacy and adverse effects associated with these therapies are partly explained by our insufficient understanding of the tissue substrate and
proarrhythmic mechanisms that are responsible for the occurrence of lethal ventricular tachyarrhythmias.

Taken together, the current treatment of cardiac arrhythmias, including means to prevent arrhythmias, is still suboptimal. It is therefore essential to better comprehend the underlying proarrhythmic 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 ischaemic 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 arrhthymias in cardiac cultures using several anti-proliferative agents, with cytochemical and extra- and intracellular electrophysiological techniques.

2. Methods

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

2.1 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 4–8 \(\times\) 10\(^3\) 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 \(\mu\)g/mL) and paclitaxel (0.085 mg/mL), which were added at Day 1 of culture and incubated for 2 h, 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 earlier, to maintain initial ratio and cell density.

2.2 Immunocytochemical 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 Supplementary material online, 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).

2.3 Western blot analyses

Homogenates were made from either three 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.

2.4 Proliferation assays

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

2.5 Apoptosis assay

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

2.6 Optical and multi-electrode mapping

At Days 4 and 9, cardiac cultures were loaded with 16 \(\mu\)mol/L Di-4-ANEPPS, given fresh DMEM/Ham’s F12 (37°C) and immediately mapped using the Ultima-L optical mapping setup (Scimedia, Costa Mesa, CA, USA). Throughout mapping experiments, cultures were kept at 37°C. Optical signal recordings were analysed 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 elminated, 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 taken in the associated data acquisition system, typically within 10 s after optical mapping. Electrograms were analysed off-line using MC-Rack software (version 3.5.6, Multi Channel Systems).

2.7 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-labelled 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 calcine AM-loaded CMCs (green), and Katushka-expressing MFBs (red) were investigated for gap-junction-mediated calcine transfer into MFBs by fluorescence microscopy.

2.8 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 \(\mu\)mol/L; TTX, Sigma-Aldrich) or verapamil (100 \(\mu\)mol/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.

2.9 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 Materials and methods can be found in the Supplementary material online.
3. Results

3.1 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 1A). Connexin43 (Cx43) was present between adjacent CMCs, MFBs, and at heterocellular junctions (Figure 1A). Dye transfer experiments demonstrated functional gap junctional MFB–CMC coupling (Supplementary material online, Figure S1). 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 and C). Of all α-SMA positive MFBs, 98.5 ± 1.6% also expressed cytoplasmic collagen-I, centred around the nucleus ($R^2 = 0.9921$) (Figure 1D and 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 and H). Immunocytochemical 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.

3.2 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 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. Days 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 1 Characterization of cardiac cell cultures at Day 9. (A) Immunocytochemical 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 high 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.
3.3 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 with control (Figure 3D and Supplementary material online, Figure S2).

Under mapping conditions, ≥60% of both treated and untreated cultures were spontaneously active at Days 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 with proliferating control cultures (~81% arrhythmias (n = 75) at Day 9, Figure 4B, F, and G and Supplementary material online, Movie 1). 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) vs. 43% (n = 23)] and Day 9 [8 (n = 37) vs. 71% (n = 35)], respectively (Figure 4A and D). An example of how ectopic activity can lead to reentrant arrhythmias is demonstrated in Supplementary material online, 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 and J). Peak-to-peak electrogram amplitude was higher in mitomycin-C treated cultures (702 ± 304 vs. 96 ± 23 μV, *P < 0.0001) (Figure 4K). Spontaneous electrical activation frequency was lowered by mitomycin-C treatment compared with control (0.28 ± 0.22 vs. 3.22 ± 0.22 Hz, *P < 0.0001) (Figure 4L).
3.4 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 mg/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, CMC 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 (R² = 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).

3.5 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. 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 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 and 10.9 ± 2.4 cm/s, respectively (P < 0.05 vs. all) (Supplementary material online, Figure S3A). 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 (Supplementary material online, Figure S3C).

Unfortunately, cultures with >50% added MFB percentages developed structural inhomogeneities from Day 6 onwards and therefore could not be studied at Day 9. Nevertheless, linear regression analysis revealed a strong inverse relationship between plated MFB percentages and CV at Day 4 (Supplementary material online, Figure S3B).
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 (4 ms isochrone spacing). Red asterisks mark ectopic foci. (B) Activation map of a reentrant tachyarrhythmia in an untreated cardiac culture (spacing: 4 ms). (C) Typical activation map of uniform conduction across a mitomycin-C treated culture (spacing: 4 ms). (D) Quantification of incidence of ectopic activity at Days 4 and 9 reveals a substantial reduction by mitomycin-C treatment. (E) CV measured by optical mapping. *P < 0.001 vs. mitomycin-C Days 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 Days 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.
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 < 0.001 vs. all including control) (Supplementary material online, Figure S3D). Furthermore, arrhythmia occurrence was 3.1-fold higher in non-treated cultures [26 (n = 17) vs. 82% (n = 23)] (Supplementary material online, Figure S3E).

3.6 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 when compared with non-reentrant conduction in cultures from the same experimental group. Furthermore, administration of TTX to 12 untreated, arrhythmic cultures at concentrations of 5 and 20 μM at Day 9 of culture resulted in a significantly lower CV (Figure 6B, D, and 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 Supplementary material online, 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 s, after which propagation resumed at a significantly lower CV of 11.5 ± 2.0 cm/s (∗∗∗P < 0.0001, 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 ongoing MFB proliferation, the maximal negative diastolic potential of CMCs was significantly reduced (∗∗∗P < 0.001, n = 12) when compared with those of CMCs in mitomycin-C treated cultures (∗∗∗P < 0.001, n = 12) (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 (∗P < 0.05, n = 8) were comparable with 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.

### 3.7 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 (Supplementary material online, Figure S4B and C) and apoptosis was not significantly increased compared with vehicle-control (0.9% DMSO)-treated cultures (Supplementary material online, Figure S4D and E). 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 (∗P < 0.05 vs. baseline, ∗∗∗P < 0.0001 vs. 0 and 5 μM). At Day 9, paclitaxel-treated cultures contained 33.3 ± 2.9% MFBs (Supplementary material online, Figure S4A, B), 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 (∗P < 0.05, n = 23). In control cultures at Day 9, CV was 6.5 ± 1.4 cm/s, with a reentry incidence of 93% (∗∗∗P < 0.0001 vs. Day 4 and control).

### 4. Discussion

Key findings of this study are (i) proliferation of MFBs in myocardial cultures results in a highly pro-arrhythmogenic substrate, in which CMCs are depolarized, conduction is slow and mainly Ca2+-driven, and ectopic activity is increased, thereby giving rise to spontaneous, sustained reentrant tachyarrhythmias, and (ii) antiproliferative treatment of these cultures prevents or substantially reduces the occurrence of arrhythmias by limiting MFBs-induced depolarization and preserving uniform, rapid, Na+-driven impulse propagation.
CMCs, with less ectopic activity, but without noticeable adverse effects on electrophysiological properties and without increased apoptosis in the treated cultures.

4.1 Myofibroblasts and cardiac arrhythmogeneity

In vitro studies indicate that MFBs could play a role in modulating electrophysiological properties in remodelled hearts, and thereby contribute to arrhythmogenesis. Rook et al. showed that cardiac fibroblasts and CMCs are able to form functional heterocellular gap junctions. Gaudeius et al., and previous studies by our group, 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. Besides their effects on CV, MFBs may also induce ectopic activity in cardiac cultures as demonstrated by Miragoli et al. Recently, a study by Zlochiver et al. showed that MFBs are able to contribute to rhythm disturbances in cardiac cultures, which was further investigated in a number of in silico studies. 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.

4.2 Reentrant tachyarrhythmias in cardiac cultures with ongoing MFB proliferation

In previous studies, functional reentry was induced by rapid electrical pacing of the cultures. 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, 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 Ca2+-channels. It is known that Ca2+-driven propagation contributes to slow conduction. In line with this observation, all arrhythmias in the present study were terminated when L-type Ca2+-channels were blocked. In contrast, most arrhythmias sustained after Na+-channel blockade, indicating that electrical propagation in such conditions appears to be mainly Ca2+-driven. Recently, Chang et al. showed similar results in another in vitro model of reentry, using non-fibroblastic cells. Moreover, as these CMCs become depolarized by increasing numbers of MFB, they could become active as local pacemaker site through depolarized-induced automaticity.

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.

4.3 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-cross-linking agent. After proliferation inhibition by mitomycin-C, CV remained stable from Days 4 to 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 in 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 neointimal hyperplasia in the myocardium. We show that paclitaxel is also suited as an 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, 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. 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.

4.4 In vivo translation

The present study provides new insights into 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 proved 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,5,28,29 and possible cardio toxic effects of antiproliferative agents,30 careful consideration of the time-frame, location and disturbances may have profound effects on arrhythmia vulnerability.

4.5 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.

4.6 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 antarrhythmic strategies.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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