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Viral vector-mediated gene transfer in fundamental and applied cardiovascular research

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Citation

Swildens, J. (2012, October 11). *Viral vector-mediated gene transfer in fundamental and applied cardiovascular research*. Retrieved from <https://hdl.handle.net/1887/19949>

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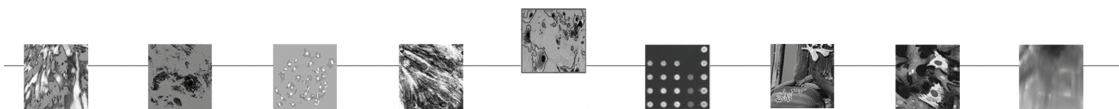


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Title: Viral vector-mediated gene transfer in fundamental and applied cardiovascular research

Issue Date: 2012-10-11



Chapter 5

Targeting of Heterocellular Coupling in Fibrotic Neonatal Rat Myocardial Cell Cultures by Lentiviral Connexin43 Knockdown in Myofibroblasts is Anti-Arrhythmic.

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* Equal contribution

Abstract

Aims: Cardiac fibrosis is associated with an increased risk of cardiac arrhythmias. However, the underlying proarrhythmic substrate remains poorly understood and the efficacy and curative potential of antiarrhythmic therapies are limited. This study aims to test the hypothesis that myofibroblasts (MFB) alter cardiomyocyte (CMC) excitability by electrotonic coupling via gap junctions leading to arrhythmias and that therefore connexin43 (Cx43) knockdown in MFBs, through lentiviral vector transduction would lower the incidence of such arrhythmias.

Methods and Results: Co-cultures of CMCs and MFBs in a 1:1 ratio as a model of cardiac fibrosis, and purified cultures of CMCs were studied for arrhythmogeneity at day 9 of culture by optical mapping using a voltage-sensitive dye. Cultures were also studied for electrophysiological properties, gap junctional coupling and protein expression. Heterocellular coupling in MFB-CMC co-cultures was reduced by transducing MFBs with a lentiviral vector encoding shRNA targeting Cx43 prior to co-culturing. In control groups, MFBs were transduced with a lentiviral vector encoding a shRNA targeting pLuc.

Conduction velocity (CV) was significantly lower in co-cultures compared to purified CMC cultures (10.9 ± 2.4 cm/s vs. 21.6 ± 3.6 cm/s $p < 0.0001$). Of the former cultures ($n=30$ total) 30.0% showed spontaneous reentrant tachyarrhythmias compared to 3.2% in the latter cultures ($n=63$ in total). CMC membrane potential was significantly more depolarized in fibrotic cultures, and was associated with lower rate of rise of depolarization (by 42.3%, $p < 0.0001$), compared to CMC cultures. Silencing of Cx43 expression in MFBs, however, increased CV by 37.5% ($p < 0.001$), reduced depolarization of adjacent CMCs and improved CMC excitability, thereby reducing the incidence of reentry by 41.1%, compared to co-cultures containing pLuc-silenced MFBs.

Conclusion: Electrotonic coupling of MFBs to CMCs in myocardial cell cultures gives rise to spontaneous reentrant tachyarrhythmias. However, targeting of this heterocellular coupling, by reducing the expression of Cx43 in MFBs, limits conduction slowing and ectopic activity, and thereby inhibits such arrhythmias. These results provide a rationale for the development of substrate-oriented anti-arrhythmic therapies.

5.1 Introduction

Acute cardiac injury such as myocardial infarction and chronic cardiac injury as seen in chronic pressure overload or in the ageing heart generally lead to cardiac fibrosis.^{1,2} Besides the detrimental consequences for the mechanical function of the myocardium, fibrosis is also closely associated with an increased risk of potentially lethal cardiac tachyarrhythmias.^{3,4} Currently, no truly curative therapeutic option is available for such fibrosis-associated arrhythmias.^{5,6} This has raised a strong incentive to identify novel therapeutic targets of such arrhythmias.

One of the hallmarks of cardiac fibrosis is a substantial increase in the relative number of myofibroblasts (MFBs) that actively remodel the myocardium.^{7,8} These MFBs increase structural heterogeneity and play a dominant role in ectopic activity and conduction slowing, which culminates in an increased propensity towards reentrant tachyarrhythmias.^{9,10} These observations could implicate MFBs as (part of) the pro-arrhythmic substrate in fibrosis-associated arrhythmias. The underlying mechanisms are based on secretion of paracrine factors or heterocellular coupling between MFBs and cardiomyocytes (CMCs), which would depolarize the CMC's resting membrane potential and would modulate ion channel availability and concomitant excitability.⁹⁻¹³ Recently, we showed that limitation of such paracrine factors and depolarization by preventing MFB proliferation has very strong anti-arrhythmic effects.¹¹ To further define the arrhythmic substrate and provide new insights in potential anti-arrhythmic targets, we investigated the effect of direct inhibition of MFB-CMC coupling on development of arrhythmias in fibrotic myocardial cell cultures by selective downregulation of Cx43 expression in MFBs. Our results demonstrate that inhibition of heterocellular coupling limits (1) MFB-induced depolarization, (2) ectopic activity and (3) conduction slowing, and consequently (4) development of spontaneous reentrant arrhythmias. This data highlight MFB-CMC coupling as a potential novel target for anti-arrhythmic therapies.

5.2 Materials and 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.

5.2.1 Cell isolation and culture

Primary neonatal rat CMCs and cardiac fibroblasts were isolated and cultured according to methods adopted from Pijnappels *et al.*¹⁴ After isolation, cell-aggregates were removed by filtering cell-suspensions through a 20 μ m cell-strainer. Next, cells were counted and plated out on fibronectin-coated round glass coverslips (15 mm) at a cell density in the range of 1×10^5 to 8×10^5 cells/well in 24-well plates (Corning Life Sciences, Amsterdam, the Netherlands), depending on the experiment.

For fibrotic co-cultures, primary CMCs and MFBs obtained from an earlier isolation were counted and mixed in a 1:1 ratio before plating. To maintain the 1:1 MFB-CMC ratio, all co-cultures were treated with the anti-proliferative agent mitomycin-C (Sigma-Aldrich,

St. Louis, MO, USA) at day 1 of culture as described previously.¹¹ In addition, purified CMC cultures were prepared and treated with mitomycin-C at day 1, which prevented the culture from becoming fibrotic. 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 37°C and 5% CO₂.

5.2.2 Characterization of cultures

Immunocytochemical analyses Following mapping experiments at day 9 of culture, cultures were stained for proteins of interest, or cultures were stained for various markers parallel to mapping experiments as described in earlier studies.¹¹ Cultures were stained for α -smooth muscle actin (α -SMA) and vimentin expression to study fibroblasts phenotype (antibodies from Sigma-Aldrich), collagen-I expression to quantify MFB numbers (antibody from Abcam, Cambridge, MA, USA), and α -actinin as CMC-specific marker (antibody from Sigma-Aldrich). In addition, cultures were also stained for connexin 43 (Cx43) (antibody from 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 μ g/mL; 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 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, 100 $\times$). All cultures were stained using the same solutions and captured using equal exposure times for the protein of interest.

5.2.3 SIN-LV production

The monocistronic SIN-LV LV.hUbiC.TurboFP635.WHVPRE, which codes for a far red-shifted mutant of the red fluorescent protein of the sea anemone *Entacmaea quadricolor* (designated TurboFP635 or Katushka¹⁵), was made with the aid of shuttle construct pLV.hUbiC.TurboFP635.WHVPRE. The shuttle plasmid pLV.hUbiC.TurboFP635.WHVPRE contains the human *ubiquitin C* gene promoter coupled to the coding sequence of TurboFP635 and the woodchuck hepatitis virus post-transcriptional regulatory element. pLV.hUbiC.TurboFP635.WHVPRE was generated by ligation of the 743-bp BstUI fragment from pTurboFP635-N (Evrogen, Moscow, Russia) to the 9.1-kb Klenow polymerase-treated XbaI fragment of pFugW.¹⁶

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.¹⁷

Vesicular stomatitis virus G-protein-pseudotyped self-inactivating human immunodeficiency virus type I vectors (SIN-LVs.) were produced by seeding six 175 cm² culture flasks with 1×10^5 293T cells per cm² in DMEM (Invitrogen) supplemented with 10% fe-

tal bovine serum (FBS, Invitrogen) and 10 $\mu\text{mol/L}$ 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/cm² 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 $\mu\text{mol/L}$ 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 825 $\times$ 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) 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* or *Katushka* expression 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 $\mu\text{g/mL}$ 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.

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5.2.4 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 at pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.5% sodiumdeoxycholate, and 0.1% SDS. Homogenates were size-fractionated on NuPage 12% Tris-Acetate NuPage gels (Invitrogen) before 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 Biotechnologies, Santa Cruz, CA, USA). To check for equal protein loading, β -tubulin (antibody from 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 bands was quantified by Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

5.2.5 Optical mapping

Action potential propagation patterns of purified CMC cultures and MFB-CMC co-cultures (8×10^5 cells/well in a 24-wells format) 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. Only spontaneously active cultures ($>60\%$) were included for further analyses. On day 9 of culture, cells were incubated for 15 ± 5 min with culture medium (DMEM/Ham's F10 + 5% HS) containing $16 \mu\text{mol/L}$ di-4-ANEPPS. After incubation, cultures were refreshed with DMEM/Ham's F12 (37°C) and subsequently mapped. Epi-illumination excitation light ($\lambda_{\text{ex}} = 525 \pm 25 \text{ nm}$) 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 ($\lambda_{\text{em}} > 590 \text{ nm}$) passed through a camera lens ($1 \times$ Plan-Apo, $\text{WD} = 15 \text{ mm}$; 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 $1.6 \times$ converging lens, resulting in a total field of view of 256 mm^2 and a spatial resolution of $160 \mu\text{m}/\text{pixel}$. During measurements, cultures were kept at 37°C . Electrical activation was recorded for at least 4 s at a rate of 500 or 167 frames/s, high-pass filtered and analyzed using Brain Vision Analyze 1008 (Brainvision Inc, Tokyo, Japan). For each pixel signals were averaged with 8 of its nearest neighboring pixels. Time points at which the rate of rise of fluorescence signal (dF/dt) was maximal were defined as the activation time point. Conduction velocity (CV) in cultures with a uniform activation pattern was calculated between two 3×3 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 and CV is a valuable parameter in determining therapeutic effects *in vitro*, all CV values for reentry and non reentry cultures were pooled within each group, unless stated otherwise.

5.2.6 Assessment of ectopic activity

All cultures underwent programmed stimulation with a unipolar electrode at 6 V for 4 s using an electrical stimulus module with corresponding software (Multichannel Systems, Reutlingen, Germany) to eliminate reentry, after which spontaneous activity could resume. Spontaneous electrical activity was recorded by optical mapping for 24 s after the programmed stimulus. Ectopic activity was defined as the presence of multiple pace-maker sites.

5.2.7 Whole-cell patch-clamp

Whole-cell patch-clamp measurements were performed in co-cultures of CMCs and MFBs at day 7-9 of culture. CMCs were identified by phase contrast microscopy and lack of fluorescence signal. Next, maximal diastolic 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). The pipette solution contained (in mmol/L) 10 Na₂ATP, 115 KCl, 1 MgCl₂, 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 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). For data acquisition and analysis, pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) was used.

5.2.8 Dye transfer

To investigate functional coupling between MFBs and CMCs, MFBs were labeled with LV.hUbiC.TurboFP635.WHVPRE (MOI 20). MFBs were kept in culture for 2 weeks and at least passaged twice before they were used in experiments. CMC cultures with a density of 1.0×10^5 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 calcein-AM (Invitrogen) diluted in HEPES-buffered salt solution (HBSS, Gibco, Grand Island, USA), which once internalized is hydrolyzed 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 calcein leakage from the cells. Katushka-labeled MFBs were subsequently plated out in a 1:1 ratio with the calcein-loaded CMCs. Bright-field and fluorescent images (at least 10 per culture) were acquired after 7 h of co-culture. 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). These experiments were subsequently conducted identically to the dye-transfer experiments described above.

5.2.9 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's *post-hoc* correction when appropriate, and expressed as mean±SD. Comparison between two groups was performed using Student's *t*-test. Differences were considered statistically significant if $p < 0.05$.

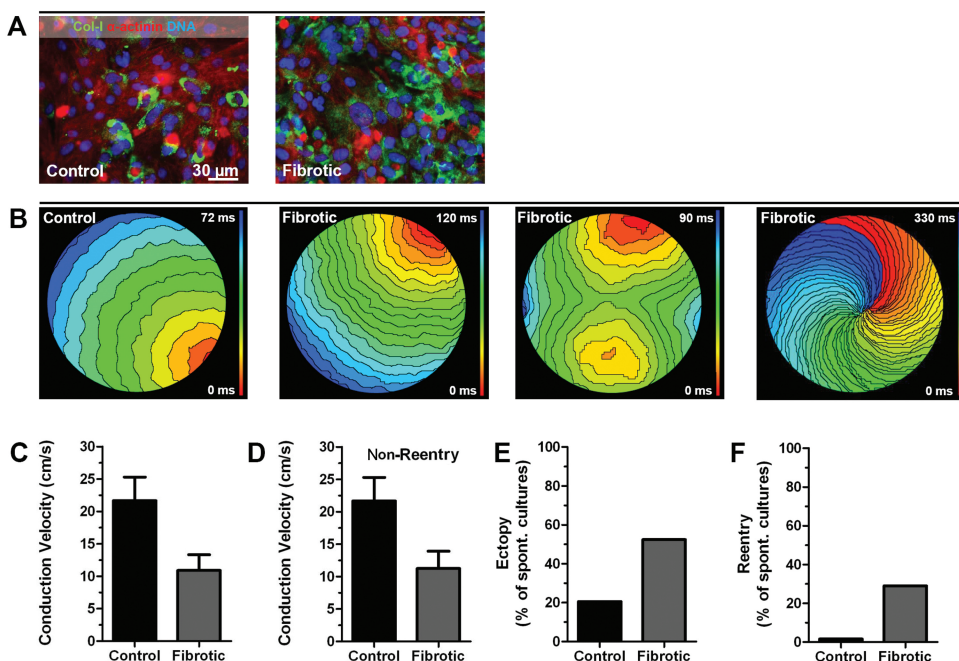


Figure 5.1: Fibrotic cultures show pro-arrhythmic tendencies. (A) Typical example of a collagen I (green) and α -actinin (red) double staining showing more MFBs in fibrotic cultures compared to control cultures. Nuclei are stained blue. (B) From left to right: typical activation map (6 ms isochrones) of purified CMC cultures showing fast propagation; typical activation map (6 ms isochrones) of slowed conduction, ectopic activity and reentry in MFB-CMC co-cultures. (C) CV in general was slowed in fibrotic cultures also when (D) reentry was excluded from analysis *: $p < 0.001$. (E) Incidence of ectopic activity was significantly higher in the fibrotic group, as was the case for (F) spontaneous reentry.

5.3 Results

5.3.1 MFBs induce ectopy and cause spontaneous reentrant tachyarrhythmias

In all cultures, $>95\%$ of all fibroblasts expressed α -SMA and collagen-I and were therefore considered as MFBs as described previously.¹¹ In fibrotic cultures, relative MFB numbers were $53.0 \pm 0.6\%$ at day 9, whereas purified CMC cultures contained $18.7 \pm 1.2\%$ 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 (10.9 ± 2.4 cm/s vs. 21.6 ± 3.6 cm/s; $p < 0.0001$ $n=30$ and $n=60$) (Figure 1B, 1C). To correct for conduction slowing attributable to reentrant conduction, CV was also

exclusively determined in non-reentry cultures. CV in these cultures was still significantly lower in fibrotic cultures than in CMC cultures (11.2 ± 2.6 cm/s vs. 21.9 ± 3.28 cm/s; $p < 0.0001$) (Figure 1D). In addition to lowered CV, fibrotic cultures were prone to develop ectopic activity (Figure 1B, second picture from the right). Of all fibrotic cultures (total $n=30$) 52.3% showed ectopic activity as opposed to 19.6% in CMC cultures (total $n=60$) (Figure 1E). Moreover the incidence of reentrant tachyarrhythmias (Figure 1B, right) was considerably higher in fibrotic cultures when compared to CMC cultures (30.0% vs. 3.2%) (Figure 1F). Together these results illustrate the arrhythmogenic potential of MFBs in cardiac cultures.

5.3.2 Electrotonic interaction between MFBs and CMCs alters CMC excitability

To study intercellular coupling between CMCs and MFBs, MFB-CMC co-cultures were stained for the gap junctional protein Cx43. Immunocytochemical staining revealed that Cx43 expression was located between adjacent MFBs, CMCs and MFBs adjacent to CMCs, suggesting the ability of MFBs to form homo- and heterocellular gap junctions (Figure 2A and B). As to the functionality of these gap junctions, we studied calcein dye transfer from CMCs to adjacent MFBs. Figure 2C shows a representative image of adjacent calcein-positive MFBs and CMCs, confirming this ability. Next, electrophysiological consequences of heterocellular coupling were assessed. The presence of MFBs in fibrotic cultures made the membrane potential of CMCs significantly more positive (-49.4 ± 6.1 mV vs. $-64.8.4 \pm 5.9$ mV; $p < 0.001$ $n=7$ and $n=8$) (Figure 2D). In agreement with the previous finding, $dF/dt_{\max}$ was significantly lower in fibrotic cultures than in CMC cultures (Figure 2E and F), which is consistent with the lower CV in these cultures.

5.3.3 shRNA-mediated Cx43 silencing by viral transduction

At MOI 32 we found maximal expression of eGFP-tag in pure MFB cultures. 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 (Figure 3A). Uniform eGFP expression was consistently confirmed by fluorescence microscopy (Figure 3B). To exclude the possibility of solely silencing the cytoplasmic or compartmentalized Cx43, Cx43 knockdown was confirmed by immunocytochemical analysis. After transduction with LV.SM2C.Cx43.hPGK.eGFP, Cx43 expression in MFB-MFB junctions was markedly lower (74%) after transduction with Cx43 silencing (Figure 3B, C).

5.3.4 Silencing Cx43 expression in MFBs reduces functional heterocellular coupling

The effect of Cx43 knockdown on functional heterocellular coupling was first assessed by calcein dye transfer experiments. The functional 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

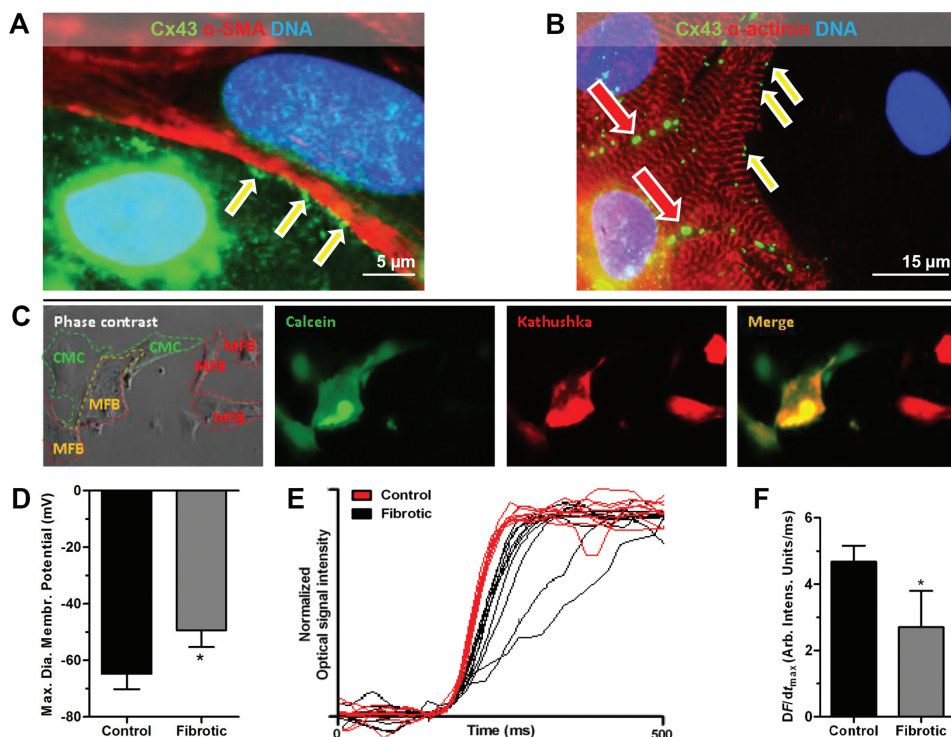


Figure 5.2: Heterocellular MFB-CMC coupling alters cardiomyocyte excitability. (A, B) Cx43 expression was present at homo- and heterocellular junctions. Red arrows indicate homocellular, CMC-CMC junctions. Yellow arrows indicate heterocellular MFB-CMC junctions. (C) Dye transfer assay shows that Katushka-labeled MFBs in direct contact with calcein-loaded CMCs exhibit transfer of the calcein, whereas isolated MFBs showed no such transfer. (D) Maximal diastolic potentials obtained from whole-cell current-clamp recordings revealed that CMCs in fibrotic cultures are significantly more depolarized as compared to CMC cultures (control). *: $p < 0.001$. (E) Upstroke traces from 10 amplitude normalized optical signals show less steep slopes of the action potential upstrokes in fibrotic cultures, indicating lowered excitability of CMCs in these cultures. (F) Quantification of the maximal upstroke velocity shows significantly lower excitability in fibrotic cultures than in CMC cultures (control). *: $p < 0.001$.

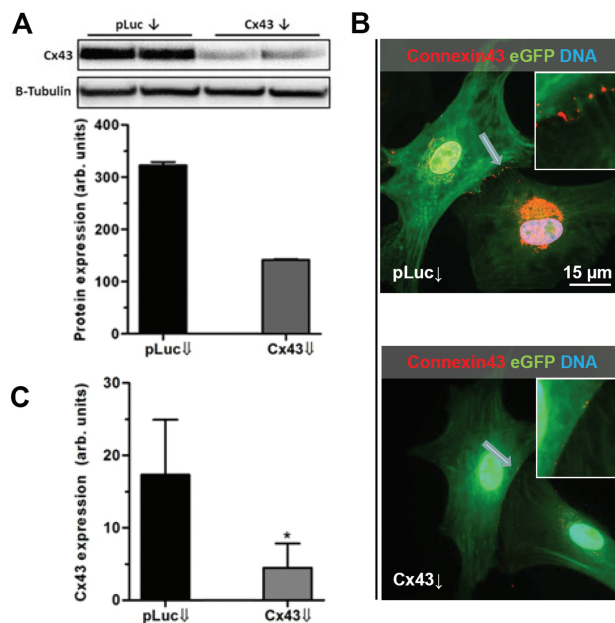


Figure 5.3: shRNA-mediated Cx43-silencing by lentiviral vectors decreases Cx43 expression in infected MFBs. (A) Typical example and quantification of Cx43 expression, normalized for β -tubulin expression from Western blot analysis showing significantly lower expression of Cx43 in Cx43-silenced MFBs compared to control ($p<0.001$). (B) Typical example of an immunocytochemical 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).

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Lv.SM2C.pLuc.hPGK.eGFP-transduced MFBs in co-cultures ($22.4\pm6.5\%$ vs. $40.8\pm10.6\%$; $p<0.01$ $n=50$ cell pairs across 7 photos per group) (Figure 4A and B). In addition, fluorescence intensity of the transferred dye was significantly lower in the Cx43 knockdown group than in the pLuc-silenced MFBs in co-cultures, also demonstrating decreased heterocellular coupling (Figure 4C). Next, CMCs in the co-cultures were analysed by whole-cell patch-clamp (Figure 4D). As a result of depressed heterocellular coupling, resting membrane potential 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; $p<0.05$) and not significantly different from the resting membrane potential in purified CMC cultures (67.5 ± 3.6 mV; n.s.) (Figure 4E and F). Relative MFB numbers, distribution and density did not differ between co-cultures with Cx43-silenced and pLuc-silenced MFBs (Figure 5A). 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 5B).

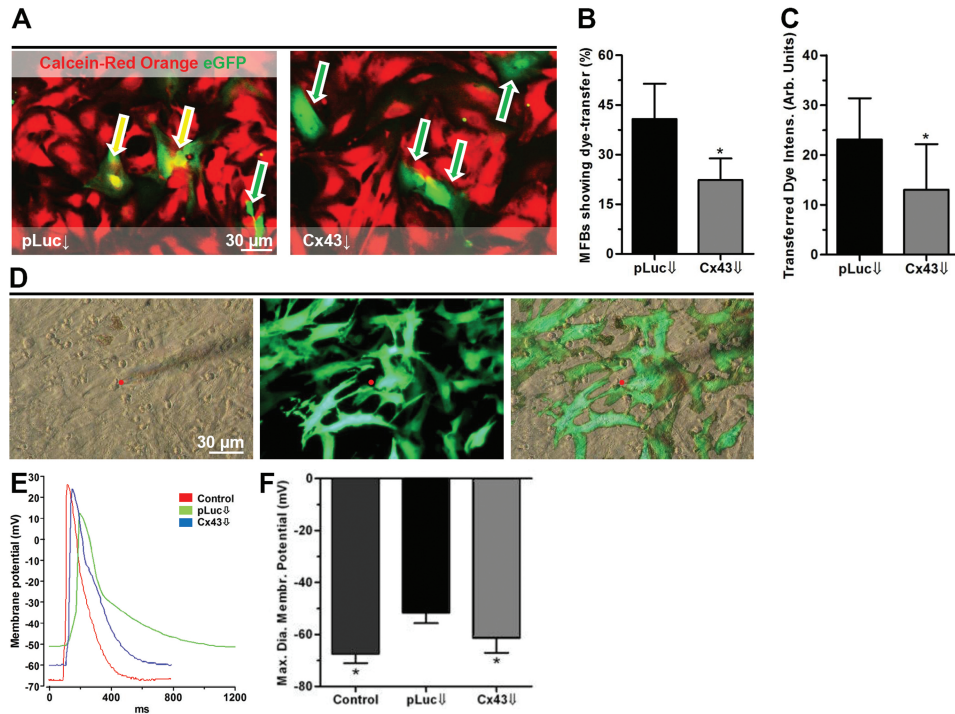


Figure 5.4: Functional consequences of MFB Cx43 silencing in CMC-MFB co-cultures. (A) Typical examples of calcein Red-Orange loaded CMCs in co-culture with eGFP expressing, Cx43-silenced MFBs or eGFP expressing, pLuc silenced MFBs. Yellow arrows indicate MFBs that show transfer after 7 h of co-culture, whereas green arrows indicate MFBs that do not receive calcein from neighboring CMCs. (B) Quantification of GFP-positive MFBs showing transfer of calcein Red-Orange per image shows significantly less cells positive for the gap junctional permeable calcein. *: $p < 0.001$ (C) Quantification of fluorescence intensity of the transferred dye shows significantly lower fluorescent signal in Cx43-silenced MFBs than in pLuc-silenced MFBs. *: $p < 0.001$. (D) Typical example of co-cultures under patch-clamp conditions showing selective targeting of CMCs surrounded by eGFP positive MFBs. Red dot signifies the tip of the electrode. (E) Typical examples of whole-cell patch-clamp recordings of CMCs in different myocardial cultures. (F) 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.

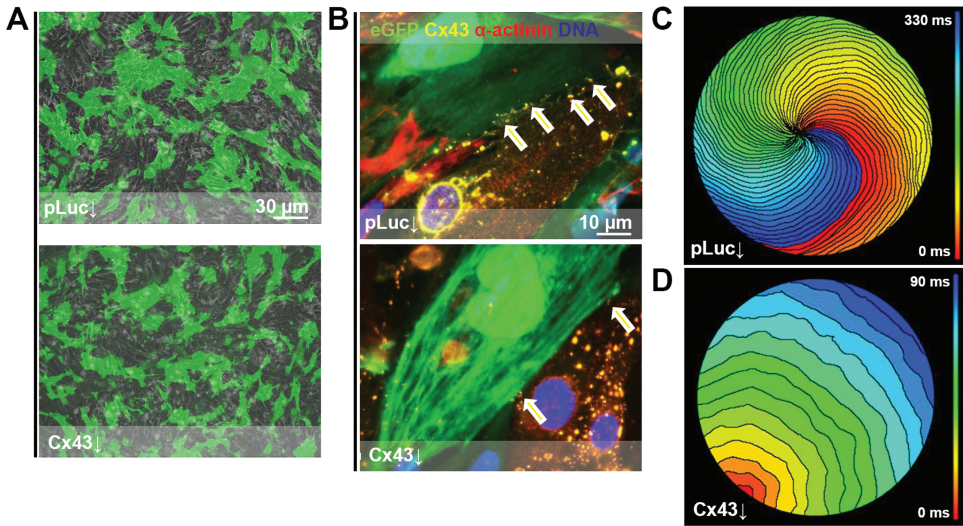


Figure 5.5: Heterocellular Cx43 expression is diminished in co-cultures with Cx43 silenced MFBs. (A) Typical example of a merged image of phase-contrast and eGFP fluorescence showing equal distribution and relative numbers of Cx43-silenced or pLuc-silenced green MFBs in co-cultures. (B) Typical example of Cx43/α-actinin staining showing decreased intercellular Cx43 expression in co-cultures with Cx43-silenced MFBs compared to co-cultures with pLuc-silenced MFBs (control). (C) Typical activation map of reentry in a co-culture with pLuc-silenced MFBs (6 ms isochrones). (D) Typical activation map of uniform propagation in a co-culture with Cx43-silenced MFBs (6 ms isochrones).

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5.3.5 Cx43 knockdown in MFBs prevents reentrant tachyarrhythmias

The anti-arrhythmogenic potential of Cx43 downregulation in MFBs was assessed by optical mapping of co-cultures. 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 6A, C). Furthermore, the incidence of ectopic activity in co-cultures was significantly decreased by Cx43 downregulation in MFBs. Of all co-cultures with LV.SM2C.Cx43.hPGK.eGFP-transduced MFBs 7.9% (total $n=38$) showed ectopy compared to 40.7% in co-cultures with pLuc-silenced MFBs (total $n=27$) (Figure 6D). Foremost, the incidence of reentrant tachycardias in co-cultures was markedly decreased by 44.1% ($n=42$) by Cx43 knockdown in MFBs compared to co-cultures with pLuc-silenced MFBs (Figure 5C, 5D and 6E). And if a co-culture with Cx43-silenced MFBs exhibited a reentrant tachycardia, its reentrant cycle length was shorter than observed in a co-culture with pLuc-silenced MFBs (Figure 6F). Together, these results demonstrate that reduction of heterocellular coupling in fibrotic myocardial cell cultures through Cx43 silencing in MFBs resulted in considerable anti-arrhythmic effects.

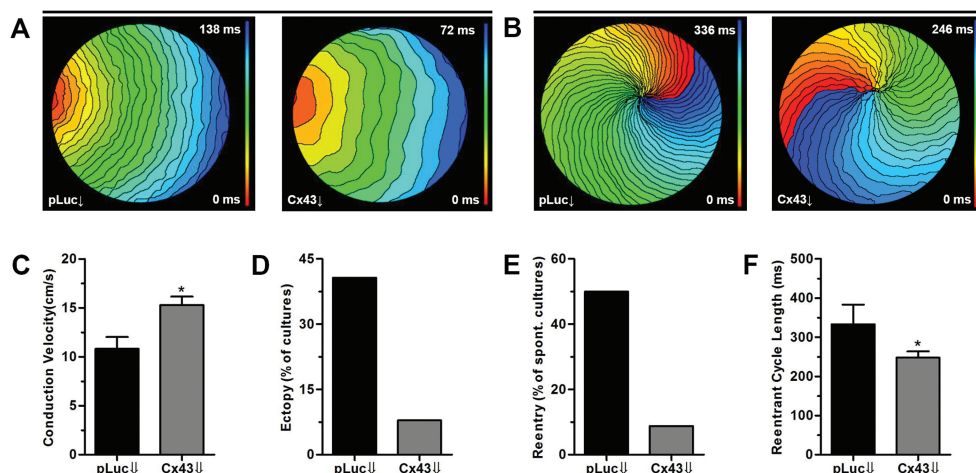


Figure 5.6: Cx43-silencing in MFBs protects the co-culture against ectopic activity and arrhythmias in fibrotic myocardial cultures. (A) Typical activation maps of uniform propagation in co-cultures with Cx43-silenced MFBs and pLuc-silenced MFBs (6 ms isochrones). (B) Typical activation maps of reentrant conduction in co-cultures with Cx43-silenced MFBs and in co-cultures with pLuc-silenced MFBs (control). (C) 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. (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). (F) Reentrant arrhythmias were significantly faster in co-fibrotic cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs. *: $p < 0.001$ vs. co-cultures with pLuc silenced MFBs.

5.4 Discussion

The key findings of this study are (1) MFBs are functionally coupled to CMCs through gap junctions and are thereby able to depolarize the CMC membrane potential through electrotonic interaction, which diminishes CMC excitability and increases the incidence of ectopic activity and spontaneous tachyarrhythmias and (2) reduction in Cx43 expression in MFBs through lentiviral vector-mediated shRNA transfer significantly decreases heterocellular coupling, leading to improvement of CMC's resting membrane potential and excitability which reduce the incidence of ectopic activity and spontaneous tachyarrhythmias.

5.4.1 MFBs and cardiac arrhythmogeneity

Cardiac fibrosis, for a long time known to impair myocardial mechanics, is currently known to be pro-arrhythmic, both *in vivo* and *in vitro*.¹⁸ Unfortunately, the exact pro-arrhythmic mechanisms are still incompletely understood which hinders the development of new anti-arrhythmic strategies. The mechanism by which cardiac fibrosis was thought to be pro-arrhythmic was that of extracellular matrix-mediated CMC detachment, which might

lead to anatomical conduction block.^{19,20} However, in recent years several studies have demonstrated that MFBs, independently of matrix deposition, could play a more crucial pro-arrhythmic role than initially thought. Among the suggested mechanisms is the secretion of paracrine factors that alter CMC excitability.¹³ In addition, electrotonic interaction between MFBs and CMCs has been shown to slow conduction, increase ectopic activity, and increase the propensity to arrhythmias,^{9,10,12,21,22} as was also confirmed by the present study. By targeting MFB proliferation, all these pro-arrhythmic aspects of the MFB can be effectively targeted at once.¹¹ However, as MFB activation and proliferation typically occur after myocardial damage, as seen for instance after myocardial infarction to secure structural integrity of the myocardium, targeting of more specific mechanisms is preferable to prevent adverse effects on the cellular make-up and myocardial structure. Therefore, we investigated whether targeting of heterocellular coupling in fibrotic myocardial cell cultures diminished arrhythmias in these cultures. Our results imply a method to prevent fibrosis-associated arrhythmias while leaving the MFBs and structural integrity of the myocardium intact.

5.4.2 Gap junctional coupling and cardiac action potential conduction

Gap junctions are intercellular channels that allow electrical communication between adjacent cells carried by ions. In the myocardium, gap junctions are indispensable for mediating rapid propagation of the electrical impulse, thereby synchronizing mechanical activity.³ Gap junctional conductance can be altered through gap junctional remodeling associated with several acquired heart diseases leading to arrhythmias. This marks gap junctions as a major player in cardiac arrhythmogeneity.²³ Gap junctions are composed of hemichannel-forming connexons, which in turn are composed of connexins. In the heart, Cx43, Cx40 and Cx45 are prevalent, with Cx43 being most prominent in ventricular myocytes.^{3,23,24} Several *in vitro* studies have suggested that Cx43 upregulation in CMCs might be an anti-arrhythmic strategy, as this could counteract gap junction remodeling and prevent consequential conduction slowing and arrhythmias.^{25,26} However, overexpression of Cx43 in CMCs might pose some difficulties in fibrotic tissue. MFBs can depolarize CMCs through gap junctional coupling as their resting membrane potential is more positive than in CMCs. As heterocellular coupling depolarizes the membrane potential, CMC's excitability may become drastically altered by coupling to MFBs which slows action potential propagation. Moreover, a substantial upstroke velocity is required as the driving force of rapid propagation and would remain diminished in a fibrotic syncytium with increased coupling. The notion that an increased heterocellular coupling might in fact amplify the depolarizing effect of MFBs on CMCs precludes an increase of Cx43 expression in fibrotic myocardium as therapeutic option. In contrast, an *in vitro* study by Kizana *et al.* suggested downregulation of Cx43 expression in CMCs as a possible anti-arrhythmic strategy, as it would focally interrupt macro-reentrant arrhythmias.²⁷ However, several *in vivo* studies of mouse models with various levels of cardiac Cx43 knockdown have revealed significantly slower impulse conduction and an increased vulnerability to arrhythmias compared to wild-type mice.²⁸⁻³⁰ In addition, CMCs show a relative redundancy of Cx43 expression, illustrated by several studies performed with heterozygous knockout mice which showed that 50% or less reduction in Cx43 expression did not result

in reduction of conduction velocity.^{31,32} Taken together, these observations demonstrate the complex nature of targeting Cx43 expression in CMCs, and suggest that further refinement is necessary for gap junction modulation to become a feasible anti-arrhythmic strategy. However, the present study shows that specific targeting of Cx43 expression in MFBs can reduce the depolarizing effect of MFBs on CMCs and act therefore anti-arrhythmic and without impairing gap junctional conductance between CMCs. Technical refinement of gene transfer, for instance by using MFB-specific promotor sequences, may improve the applicability of this approach.

5.4.3 MFB dependent depolarization of CMCs and arrhythmias

Several *in vitro* studies indicate that MFB-CMC coupling could contribute to arrhythmogenesis by altering electrophysiological tissue characteristics. Functional heterocellular coupling was first observed by Rook *et al.*, and was later shown to cause slow conduction through electrotonic interaction.³³ In addition, heterocellular coupling might account for the mechanism by which cardiac excitability is affected in fibrotic tissue.^{7,9,14} These results have been confirmed in our present study showing dye transfer between MFBs and CMCs, and depressed action potentials in CMCs of MFB-CMC co-cultures, which can be attenuated by silencing Cx43 expression in MFBs. Furthermore Vasques *et al.* have demonstrated that after cardiac injury heterocellular interactions are enhanced as a consequence of a phenotypic switch of fibroblasts to MFBs.³⁴ In addition, Lau *et al.* have shown that overexpression of skeletal muscle Na⁺-channel Skm1 in depolarized myocardial infarct border zones suppresses ventricular arrhythmias. These ventricular arrhythmias are attributed to the depolarized resting membrane potential of CMCs in the infarct borderzone leading to decreased CMC excitability and lowering of CV. As Skm1 operates more effectively at a depolarized membrane potential, arrhythmias are prevented.³⁵ These studies, therefore, imply a role for targeting depolarization of CMC's resting membrane potential, possibly through MFB-CMC coupling, to prevent cardiac arrhythmias. However targeting depolarization-induced lowering of CMC excitability by overexpression of Skm1 only targets one factor altered by depolarization namely I_{Na} . In the present study we show a way of targeting depolarization of the CMC membrane potential itself, as opposed to focusing on its consequences. This was achieved by targeting of Cx43 expression in MFBs which thereby prevents the incidence of reentrant tachyarrhythmias. MFB Cx43 silencing could therefore be a promising approach in the treatment of fibrosis-associated arrhythmias.

5.4.4 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 Cx43 was targeted to reduce heterocellular coupling between CMCs and MFBs. However, in addition to Cx43, MFBs also express Cx40 and Cx45.¹⁶ Heterocellular gap junctions through Cx40 and 45 are not inhibited by this intervention and could therefore still contribute to MFB-induced depolarization of adjacent CMCs. However, considering

the relative expression of Cx43 opposed to Cx40 and Cx45 in CMCs as well as in MFBs, and the marked effect of Cx43 silencing on dye transfer suggests that the roles of Cx40 and Cx45 on electrical coupling of MFBs with CMCs are of minor importance.

5.4.5 Conclusions

MFBs are able to form functional heterocellular gap junctions with CMCs and are therefore able to (1) diminish CMC excitability, (2) reduce CV in cardiac cultures, (3) induce ectopic activity, and (4) give rise to spontaneous reentrant tachyarrhythmias in fibrotic myocardial cell cultures. MFB-CMC coupling can be attenuated by selective silencing of Cx43 expression in MFBs using lentiviral vector mediated shRNA technology. Moreover, targeting heterocellular gap junctional coupling preserves CMC excitability, limits conduction slowing and the incidence of ectopic activity and thereby prevents reentrant tachyarrhythmias. Thus, this study provides a rationale for new therapies of cardiac arrhythmias, based on a selective, substrate-oriented approach.

5.4.6 Funding

This work was supported by the Dutch Heart Foundation (2008/B119). D.A.P. is the recipient of the Netherlands Organisation for Scientific Research (NWO) VENI grant.

5.5 Acknowledgements

We thank Dr. Wilbert P.M. van Meerwijk for constructive discussions, and Huybert J.F. van der Stadt for excellent technical support.

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