Cellular and Molecular Mechanisms of Arrhythmias in Cardiac Fibrosis and Beyond:

From Symptoms to Substrates towards Solutions

Chapter VII

Forced Cellular Fusion of Human Ventricular Scar Cells with Neonatal Rat Cardiomyocytes Ameliorates Their Arrhythmicity

Saïd F. A. Askar, MSc; Brian O. Bingen, MD; Martin J. Schalij, MD, PhD; Zeinab Neshati, MSc; Michel I.M. Versteegh, MD; Robert J.M. Klautz, MD, PhD; Jerry Braun, MD, PhD; Antoine A. F. de Vries, PhD*; Daniël A. Pijnappels, PhD*.

*Equal contribution

Abstract

Aims: Fibroblasts can be pro-arrhythmic due to their detrimental effects on cardiomyocyte electrophysiology through several mechanisms that rely on suboptimal integration into the cardiac syncytium. To force full integration of fibroblasts into the cardiac syncytium, the feasibility of heterocellular fusion between fibroblasts and cardiomyocytes as a novel anti-arrhythmic strategy was investigated.

Methods & Results: Human ventricular scar cells (hVSC) were isolated from human ventricular post-myocardial infarction scars. Co-cultures of eGFP- or Vesicular-Stomatitis-G-protein- and eGFP-expressing hVSCs and neonatal rat cardiomyocytes (nrCMCs) were prepared in a 1:4 ratio. Fusion was induced by brief exposure to acidic buffer (pH6.0) at day 3 and electrophysiological effects of fusion were evaluated at day 5 by optical mapping. Simultaneous expression of fibroblast-specific collagen-I and nrCMC-specific α-actinin was observed in multinucleated cells that contained both human and rat nuclei, without increased apoptosis as judged by annexin V staining. These nrCMC-hVSC heterokaryons retained sarcomeric α-actinin expression and remained contractile, while vimentin expression in nrCMC-hVSC heterokaryons was lower than in non-fused hVSCs. Moreover, Cx43 and Cav1.2 protein levels were increased in heterokaryons compared to unfused hVSCs. Fused cultures showed faster conduction (16.8±1.6 vs. 10.3±2.6 cm/s, $P<0.05$) and shorter action potential duration (328±56 vs. 480±79ms, $P<0.05$). Triggered activity and reentry were ameliorated by heterocellular fusion (incidences of 63%, n=8 vs. 0%, n=23 in fused co-cultures).

Conclusion: Heterocellular fusion between hVSCs and nrCMCs is feasible and well-tolerated as it forms contractile heterokaryons. Importantly, heterocellular fusion had strong anti-arrhythmic effects. As the nrCMC phenotype appeared to be dominant within the heterokaryon, these results may provide novel insights in anti-arrhythmic reprogramming of fibroblasts.

Introduction

Despite the well-established pro-arrhythmic role of cardiac fibrosis, its mechanisms remain incompletely understood. Although the extracellular matrix-synthesizing fibroblasts have long been known to be key effectors of fibrosis, it has only relatively recently become apparent that fibroblasts themselves can contribute to arrhythmogeneity through several mechanisms.[1-3] These unexcitable cells are abundantly present after pro-fibrotic myocardial injury such as myocardial infarction. Such fibroblastic ventricular scar cells (VSCs) form clusters and intersperse between cardiomyocytes, which due to their inexcitability and poor gap-junctional coupling to cardiomyocytes, anatomically obstructs conduction and contributes to pro-arrhythmogeneity. Moreover, their membrane is more depolarized compared to cardiomyocytes, which leads to pro-arrhythmic effects by heterocellular gap-junctional coupling.[2] Therefore, targeting fibroblast integration may be too complex to be broadly applicable as an anti-arrhythmic strategy if fibroblast inexcitability is not overcome.[4] Moreover, pro-arrhythmic mechanisms of fibroblasts based on paracrine factors and mechanical coupling are unlikely to be affected by such an approach.[1,3] Therefore, alternative strategies of modifying electrical fibroblast integration need to be explored that may offer a radically different, but effective approach.

In regenerative medicine, cellular fusion has been studied mostly as an infrequently occurring spontaneous phenomenon between stem cells and cardiomyocytes in the context of cellular (de-)differentiation.[5,6] However, its effects have never been experimentally studied in the context of fibrosis-associated arrhythmias. Heterocellular fusion between fibroblasts and cardiomyocytes may not only promote full electrical integration of fibroblasts with cardiomyocytes, but also may limit all of their pro-arrhythmic mechanisms as cellular properties can be altered by cellular fusion.[7] Thereby, cellular fusion may provide a novel strategy to target the pro-arrhythmic effects of fibroblasts. In the current study, we therefore investigated the effects of cellular fusion between human ventricular scar cells (hVSCs) and neonatal rat cardiomyocytes to explore the concept of heterocellular fusion as a strategy to limit pro-arrhythmic effects exhibited by hVSCs *in vitro*.

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.

Cardiomyocyte isolation and culture

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center (LUMC) and conform to the Guide for the Care and Use of Laboratory Animals, as stated by the US National Institutes of Health[7] (permit number 10236). Ventricular cardiomyocytes (nrCMCs) were isolated from neonatal Wistar rats and cultured as described previously.[8] In brief, hearts were rapidly excised from animals after confirmation of adequate anesthesia with 4-5% isoflurane and subsequently minced into small pieces. After two sequential digestion steps with collagenase I (450 units/mL; Worthington, Lakewood, NJ, USA), a pre-plating step was performed to minimize neonatal rat fibroblast contamination of the myocardial cell suspension. Myocardial cells were plated on fibronectin-coated cover slips in 24-wells plates (Corning Life Sciences, Amsterdam, the Netherlands) at a density of $5x10^5$ cells/well. To stop proliferation and maintain initially plated cell population ratios, cultures were treated with 10 µg/mL mitomycin-C (Sigma-Aldrich, St. Louis, MO, USA) at day 1 of culture for 2 h.

Human Ventricular Scar Fibroblast isolation

All human-derived tissues were collected in accordance to guidelines posed by the Medical Ethics committee of the LUMC and adhered to the principles described in the Declaration of Helsinki. Human Ventricular Scar Cells (hVSCs) were isolated from human myocardial scar tissue of 6 different post-myocardial infarction patients that was excised during the surgical Dor procedure. After dissection into small pieces, the tissue was placed in 0.1% gelatin coated six-wells plates (Corning) and covered by glass cover slips to prevent floating of the tissue. Culture plates were refreshed twice a week with culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Breda, the Netherlands) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 µg/mL penicillin and 100 µg/mL streptomycin (Bio-Whittaker, Carlsbad, CA, USA). Cells grew out for 2-4 weeks, and were trypsinized and passaged 3-6 times before use in experiments. During cell culture, hVSCs were refreshed twice a week.

Co-cultures of hVSCs and cardiomyocytes were prepared by counting and mixing the cell suspensions in ratios of 1:4 and plated out in a cell density of $2\text{-}5.0x10^5$ cells per well depending on the experiment.

Lentiviral Viral Vector production

Self-inactivating human immunodeficiency virus type I vectors were produced in 293T cells as described previously.[2] In brief, these cells were transfected with a lentiviral vector shuttle plasmid together with psPAX2 (Addgene, Cambridge, MA, USA) and pLP/VSVG (Invitrogen) using 25-kDa linear polyethyleneimine (Polysciences, Warrington, PA, USA) as transfection agent. After 16 hours, transfection medium was replaced by culture medium and after 64 hours, the culture fluid was collected by centrifugation and freed of cellular debris by filtration. Concentration of lentiviral vector particles was performed by ultracentrifugation through a 20% (w/V) sucrose cushion. Pellets containing vector particles were suspended in phosphate-buffered saline with 1% bovine serum albumin fraction V (Sigma-Aldrich).

Viral Transduction

One day prior to transduction, hVSCs were trypsinized and plated onto a 0.1% gelatin-coated six-well plate at a density of 4×10^5 cells per well. Cells from different patients were pooled for standardization and reproducibility. Cells were subsequently exposed overnight to an inoculum of 800 µL culture medium containing 10 µg/ml DEAE dextran and lentiviral particles of either LV.hCMV-IE.VSV-G.IRES.eGFP.hHBVPRE or LV.IRES.eGFP.hHBVPRE as control. Following transduction, cultures were rinsed three times with PBS and kept on culture medium until usage in experiments.

Induction and assessment of fusion

To induce fusion, a recently developed technique termed V-fusion was utilized.[10] This technique uses transgenic expression of the Vesicular Stomatitis Virus – G (VSV-G) protein which normally is involved in viral entry into mammalian cells. Fusogenic potency of this protein is increased by exposure to acidic solutions. Therefore, VSV-G expressing hVSCs in confluent co-culture with nrCMCs were exposed to CMC culture medium adjusted to pH 6.0 using 0.5 mol/L 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES, Sigma-Aldrich) during 3-5 minutes at 37^{o}C at day 3 of culture. All cultures, including cultures containing control hVSCs that solely expressed eGFP and control cultures without hVSCs were identically treated with acidic buffer.

Fusion was assessed using immunocytological staining for co-expression of the normally mutually exclusive cytoplasmic expression of collagen-I (hVSC-specific) and α-actinin (nrCMC specific) as well as the presence of multiple nuclei, positive (of human origin) or negative (of rat origin) for human-specific Lamin A/C. Fused cells were defined as multinucleated cells as judged by cytoplasmic collagen-I expression with nuclear lacunae, whereas heterokaryons were defined as multinucleated cells that also contained at least 1 human nucleus and 1 rat nucleus.

Immunocytological characterization

Co-cultures were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100 for 20 min, and stained with primary antibodies. Permabilization for >15 min resulted in loss of eGFP signal. Primary antibodies specific for human lamin A/C (Vector laboratories, Burlingame, CA, USA), cardiac α-actinin, (all Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) and collagen type I (Abcam, Cambridge, MA, USA) were used to distinguish between hVSCs and CMCs. Antibodies specific for Connexin43 (Cx43) or α-smooth muscle actin (αSMA) and N-cadherin were used to investigate expression of proteins involved in electrical or mechanical coupling, respectively. Primary antibodies were visualized with Alexa fluor-conjugated antibodies (Invitrogen), or with biotin-labeled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Qdot 655-streptavidin conjugates (Invitrogen). Nuclei were stained using Hoechst 33342 (Invitrogen). A fluorescence microscope equipped with a digital camera (Nikon Eclipse, Nikon Europe, Badhoevedorp, The Netherlands) and dedicated software (NIS Elements, Nikon and Image J, version 1.43; Institutes of Health, Bethesda, MD) were used to analyze the data. All cultures were treated equally using the same antibody dilutions and exposure times. Quantification of average pixel intensity from pictures of fluorescent staining was performed in areas of interest of a fixed size per cell that showed maximal fluorescent intensity to compare the uppers limit of expression potential of the cells in question. Quantification was performed on cells within fused co-cultures, as these cultures contained unfused hVSCs, unfused nrCMCs and heterokaryons and therefore facilitate the highest degree of comparability. In addition, quantifications in unfused co-cultures showed that the expression values of unfused hVSCs and unfused nrCMCs in these cultures was not significantly different from the expression levels found in the same cell types in fused co-cultures (data not shown).

Optical mapping

Investigation of electrophysiological parameters at the tissue level were performed with optical mapping as described previously.[11] In brief, cultures were loaded with 6 μmol/L of the voltage-sensitive dye di-4-ANEPPS (Invitrogen) diluted in DMEM/HAMS F12 (Invitrogen) for 10 min in a humidified incubator at 37°C. Cultures were refreshed with DMEM/HAMS F12 and optically mapped using the Micam Ultima-L optical mapping system (Scimedia, Costa Mesa, CA, USA). Optical signals were recorded and subsequently analyzed using Brainvision Analyze 1108 (Brainvision Inc, Tokyo, Japan). Cultures were electrically stimulated with a 10 ms pulse at ≥1.5x diastolic threshold and paced at 1-2Hz (Multichannel systems, Reutlingen, Germany) to determine conduction velocity (CV) and action potential duration (APD_{90}), as well as dispersion of repolarization. Arrhythmic episodes of reentrant tachyarrhythmias and early-afterdepolarizations were quantified during spontaneous activity or following 1Hz pacing. Dispersion of repolarization was

calculated as the maximal spatial difference in APD_{90} within the same culture during the same activation wave. The size of areas of dispersion was at least 4x4 pixels.

Statistical Analyses

Experimental results were expressed as mean±standard deviation (SD) for a given number (n) of observations. Data was analyzed by Student's t-test for direct comparisons. Analysis of variance followed by the appropriate *post-hoc* analysis was performed for multiple comparisons. Statistical analysis was performed using SPSS 16.0 for Windows (SPSS, Chicago, IL). Differences were considered statistically significant at $P<0.05$.

Results

hVSCs are highly pro-arrhythmic in co-culture with nrCMCs

CMCs and hVSCs were characterized with immunocytological staining. Human VSCs were all positive for collagen-I (n=506 cells) and vimentin (n=713 cells), but were negative for α-actinin. Cardiomyocytes were defined as α-actinin positive, and were negative for collagen-I (n=645 cells). Purified neonatal rat ventricular monolayers consisted of 12.6±3.9% neonatal rat fibroblasts and 87.4±3.9% CMCs as determined by collagen I/α-actinin staining as described previously.[12] Fibrotic co-cultures of nrCMCs and hVSCs contained 24.4±5.8% hVSCs as determined by human-specific Lamin A/C staining. At day 5 of culture, optical mapping revealed that CV was severely decreased by the presence of hVSCs (11.1±2.7 cm/s vs. 23.4±0.6 cm/s in control ($P<0.05$, Figure 1 A-C). In addition, pseudo-voltage traces showed a distinctly different action potential morphology (Figure 1D), signified by an increase in APD_{80} (349±52 ms vs. 259±25 ms in controls, $P<0.05$, Figure 1E). In addition, spatial dispersion of APD_{90} was increased by hVSCs (103±33 ms vs. 38±19 ms, $P<0.05$, Figure 1F).

Importantly, hVSCs were found to be highly pro-arrhythmic. Arrhythmic episodes, defined as early afterdepolarizations and reentrant arrhythmias could be observed frequently in the hVSC group (22 out of 25 cultures) but were rarely observed in the control group (1 out of 25 cultures, Figure 1G).

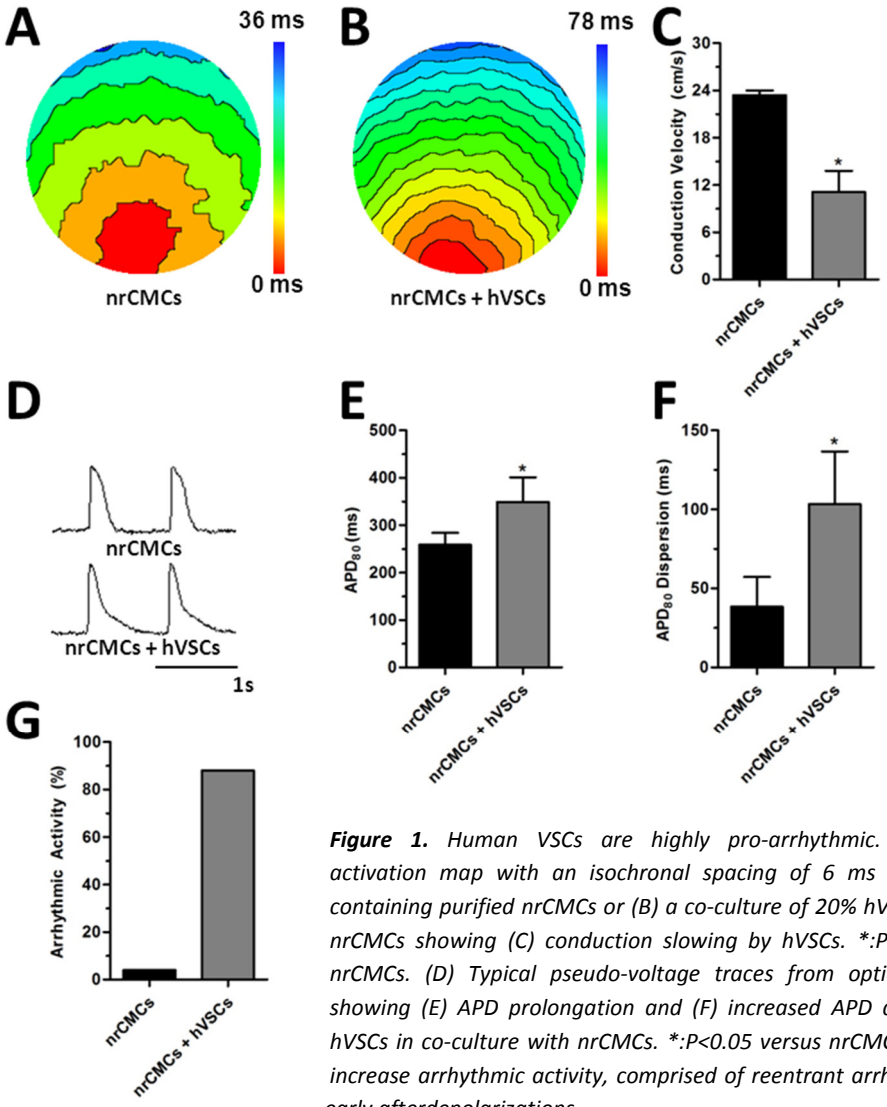

Figure 1. *Human VSCs are highly pro-arrhythmic. (A) Typical activation map with an isochronal spacing of 6 ms of a culture containing purified nrCMCs or (B) a co-culture of 20% hVSCs and 80% nrCMCs showing (C) conduction slowing by hVSCs. *:P<0.05 versus nrCMCs. (D) Typical pseudo-voltage traces from optical mapping showing (E) APD prolongation and (F) increased APD dispersion by hVSCs in co-culture with nrCMCs. *:P<0.05 versus nrCMCs. (G) hVSCs increase arrhythmic activity, comprised of reentrant arrhythmias and early afterdepolarizations.*

The V-fusion protocol leads to well-tolerated heterocellular fusion between hVSCs and nrCMCs

To induce cellular fusion without affecting intracellular membranes, a technique named V-fusion was utilized that was mediated by lentiviral VSV-G overexpression and subsequent fusion induction by brief exposure to acidic (pH=6.0) buffer. Overexpression of VSV-G was confirmed by immunocytological staining, and absence of VSV-G was confirmed in control hVSCs expressing eGFP (Figure 2A). Exposure to acidic buffer induced cellular fusion in VSV-G expressing hVSCs (Figure 2B).

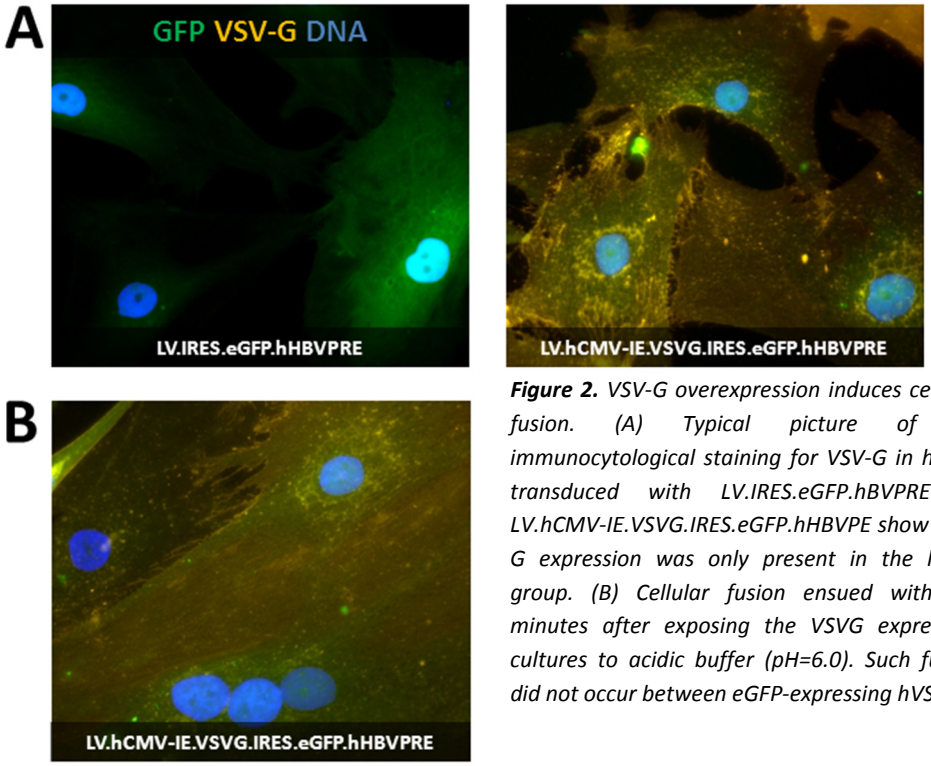

Figure 2. *VSV-G overexpression induces cellular fusion. (A) Typical picture of an immunocytological staining for VSV-G in hVSCs transduced with LV.IRES.eGFP.hBVPRE or LV.hCMV-IE.VSVG.IRES.eGFP.hHBVPE show VSV-G expression was only present in the latter group. (B) Cellular fusion ensued within 5 minutes after exposing the VSVG expressing cultures to acidic buffer (pH=6.0). Such fusion did not occur between eGFP-expressing hVSCs.*

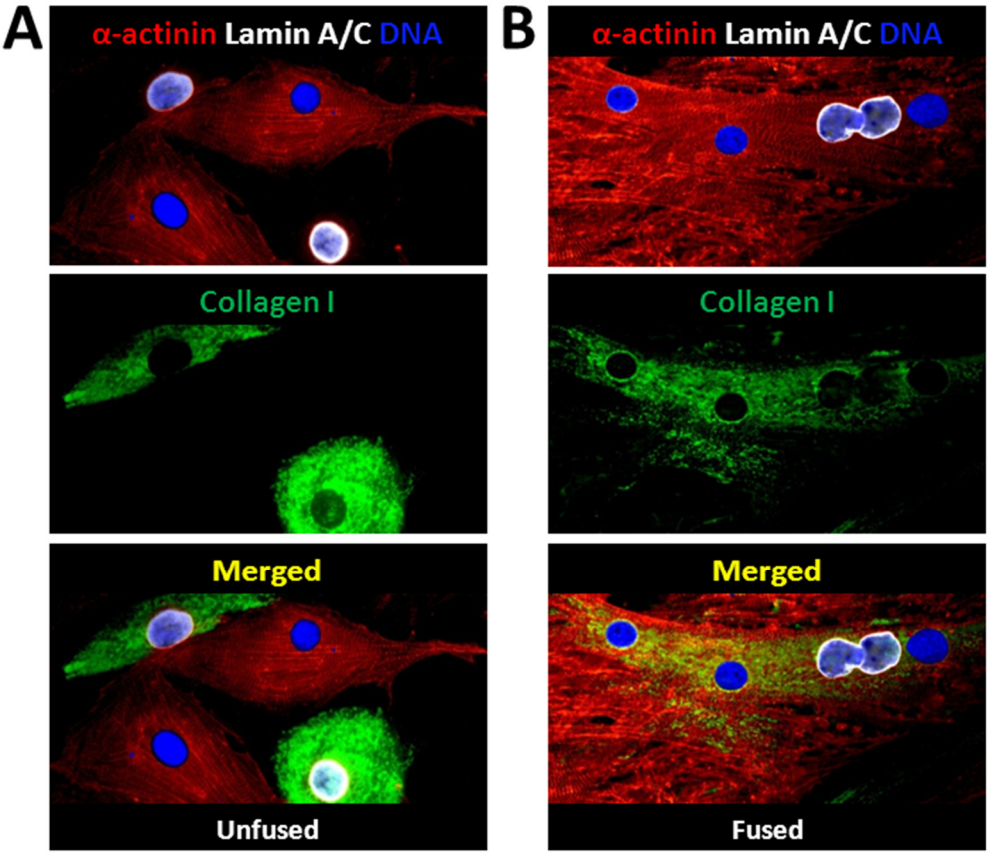

Figure 3. *Forced heterocellular fusion of nrCMCs and hVSCs leads to formation of hybrid heterokaryotic cells. (A) Typical picture of an immunocytological staining for α-actinin, lamin A/C and DNA of an unfused co-culture. For unfused cells, collagen-I expression is normally specific to hVSCs and not found in nrCMCs, while α-actinin is specific to nrCMCs and never found in hVSCs. (B) By forced heterocellular fusion, both of these markers were co-expressed within the same cell. Moreover, human nuclei (Lamin A/C positive) and rat nuclei (Lamin A/C negative) were shown to be present within the same cytoplasm as evidenced by the nuclear cavities seen in cytoplasmic collagen I expression.*

Next, co-cultures containing VSV-G expressing hVSCs and nrCMCs (from here on referred to as “fused co-cultures”) or solely eGFP expressing hVSCs and CMCs in a 1:4 ratio (henceforth referred to as unfused co-cultures) were prepared. Effects of heterocellular fusion were characterized at day 5 by immunocytological staining for human-specific Lamin A/C, Collagen-I, α-actinin and DNA. In unfused co-cultures, hVSCs expressed collagen-I and did not show positive staining for α-actinin (Figure 3A). Moreover, there were no human-specific Lamin A/C-expressing nuclei observed in α-actinin expressing

cells. However, fused co-cultures did contain heterokaryons, which were defined as cells that contained at least 2 nuclei of different species within the same cytoplasm.

Such heterokaryons co-expressed collagen-I and α-actinin and contained multiple nuclei of human and rat origin (Figure 3B). In fused co-cultures, 70±9% of human nuclei were present inside multinucleated cells, while 1±2% of human nuclei in unfused co-cultures were present within multinucleated cells. The collagen-I staining also stains cytoplasmic pro-collagen I, which produces nuclear cavities in the staining profile and, when combined with human-specific lamin A/C and Hoechst staining, allowed to identify nuclei from different species within the same cytoplasm (Figure 3). On average, heterokaryons consisted of 6±3 nuclei per cell (range of 2-17). Of these nuclei, 46±18% was positive for lamin A/C and therefore originated from hVSCs (range of 13-83%). In unfused co-cultures, 7 multinucleated cells were found, of which 6 contained only human nuclei. Moreover, all these cells contained 2 nuclei. Analysis of nuclear composition of heterokaryons (n=45) showed that 13 heterokaryons contained more than 50% human nuclei, while 10 heterokaryons contained exactly 50% human nuclei. Concomitantly, 22 heterokaryons contained more nrCMC nuclei than human nuclei. There were 3 fused cells (of 48 fused cells) that were comprised of only human nuclei. Annexin-V staining revealed no increases in apoptosis by forced cellular fusion (1.3±1.3% in eGFP controls versus 0.9±1.3% in VSV-G co-cultures, $P>0.05$), indicating that heterocellular fusion was well-tolerated.

Cytoskeletal reorganization after heterocellular fusion

To investigate cytoskeletal composition after heterocellular fusion, vimentin expression was quantified by immunocytological staining. In unfused nrCMCs, vimentin expression was low whereas vimentin expression was high in unfused hVSCs (Figure 4A, B). Interestingly, heterokaryons showed a substantial decrease in vimentin expression compared to unfused hVSCs (Figure 4B). Moreover, vimentin expression did not correlate ($r^2=0.05$) to the percentage of nuclei of human origin within the heterokaryon (Figure 4C). The contribution of nrCMC-specific cytoskeletal protein to the cytoskeleton of the heterokaryon appeared more dominant, as quantification of α-actinin staining revealed that sarcomeres remained intact in the majority of heterokaryons. More specifically, 89% of 46 heterokaryons expressed sarcomeric α-actinin, while none out of 8 fused cells comprised of only hVSCs expressed sarcomeric α-actinin. Moreover, hybrid cells remained contractile as observed under phase-contrast light microscopy.

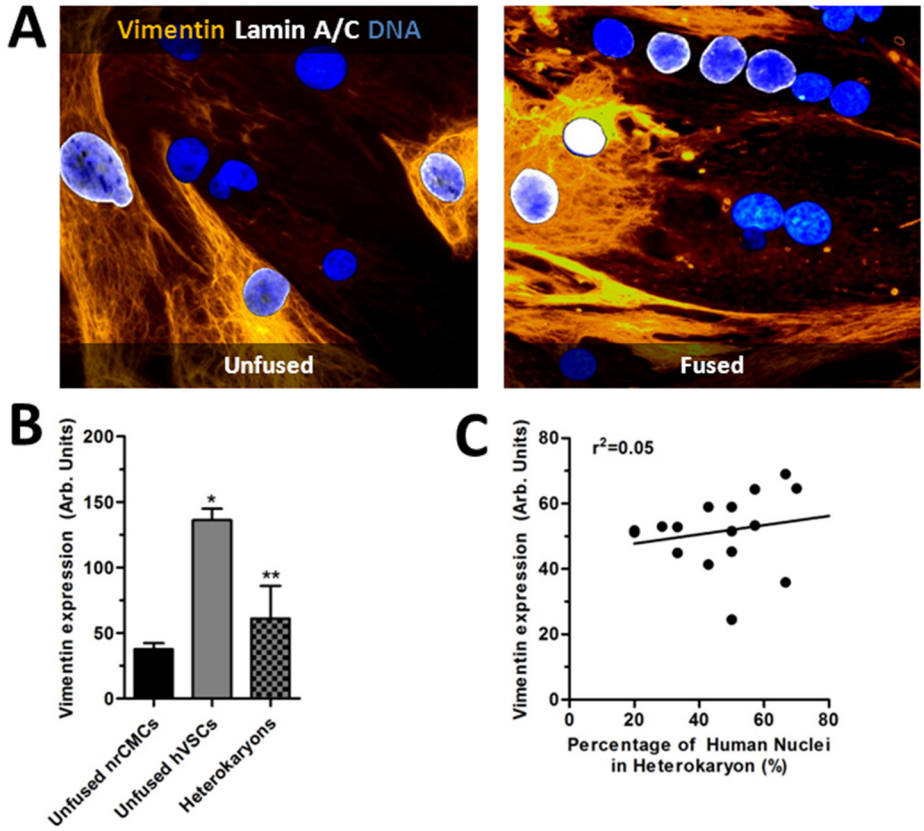

Figure 4. *Cytoskeletal rearrangement after cellular fusion. (A) Typical example of a vimentin staining showing Lamin A/C positive unfused hVSCs that are strongly positive, whereas unfused nrCMCs express lower amounts of vimentin. (B) Quantification of vimentin expression levels based on immunocytological staining. *:P<0.05 vs. fused cells and nrCMCs. **:P<0.05 vs. unfused hVSCs and nrCMCs. (C) Vimentin expression levels weakly correlated with the percentage of human nuclei within the heterokaryon.*

Effects of forced heterocellular fusion on gap-junctional protein and ion channel expression levels

To investigate whether expression of proteins involved in electrophysiology were affected by heterocellular fusion, fused and unfused cultures were stained for Cx43, the dominant cardiac gap-junctional protein. This staining revealed that while unfused hVSCs expressed low amounts of intercellular Cx43, unfused nrCMCs expressed high amounts of Cx43 at intercellular junctions (Figure 5A, B). Moreover, heterokaryons expressed Cx43 at levels higher than hVSCs and similar to nrCMCs (Figure 5B). Interestingly, Cx43 expression levels did not linearly correlate in the slightest to the amount of human nuclei present within the heterokaryon (r^2=0.05, Figure 5C). To investigate ion channel expression in heterokaryons, staining were performed for Cav1.2, the pore-forming unit of the L-type calcium channel, which was strongly expressed in unfused nrCMCs but barely detectable in unfused hVSCs (Figure 6A). Interestingly, Cav1.2 was also expressed in fused heterokaryons (Figure 6B), and in higher quantities than in unfused hVSCs (Figure 6C). Expression levels of Cav1.2 did not linearly correlate (r^2=0.02) to the amount of human nuclei present within heterokaryons, showing positive staining even when human nuclei outnumbered rat nuclei 1:4 (Figure 6D).

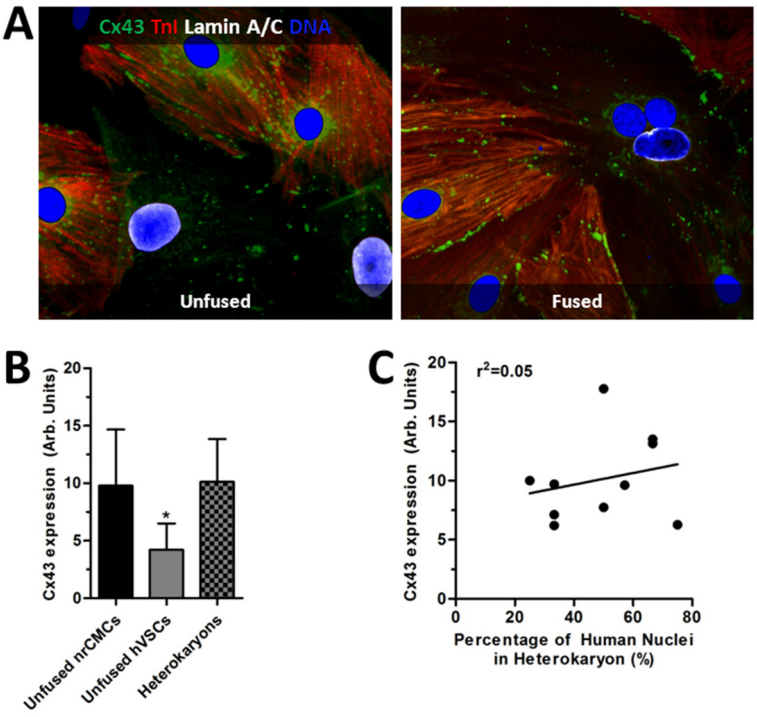

Figure 5. *Cx43 expression in heterokaryons. (A) Typical examples of Cx43 staining on unfused and fused cells showing (B) cx43 expression levels in heterokaryons was equal to those of CMCs. *:P<0.05 vs. all. (C) Cx43 expression levels in heterokaryons does not linearly depend on the amount of human nuclei present within heterokaryons.*

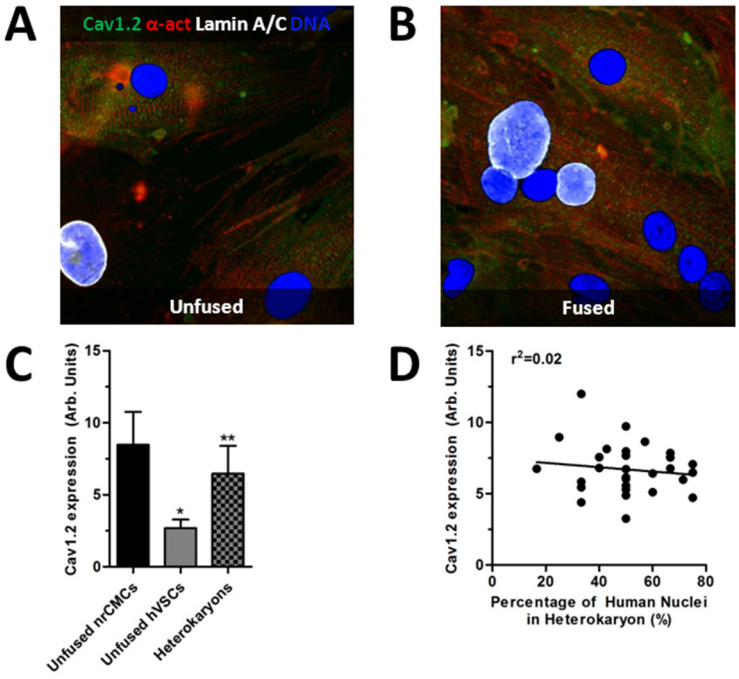

Figure 6. *Heterocellular fusion leads to Cav1.2 expression in heterokaryons. (A) Typical examples of immunocytological staining for Cav1.2 in unfused and (B) fused hVSCs showing (C) increased Cav1.2 expression levels in heterokaryons compared to hVSCs.*:P<0.05 vs. nrCMCs and fused cells. **:P<0.05 vs. CMC and No-fusion. (D) Cav1.2 expression levels did not linearly depend on the amount of human nuclei present within the heterokaryon.*

Forced cellular fusion of hVSCs and CMCs is anti-arrhythmic

Electrophysiological effects of heterocellular fusion were evaluated at day 5, 2 days after induction of fusion with the acidic buffer. Importantly, this buffer did not affect CV or APD_{90} within 4 hours after exposure or 2 days after exposure in purified CMC cultures (data not shown). By induction of heterocellular fusion, CV was significantly higher than in unfused co-cultures (16.8±1.6 cm/s vs. 10.3±2.6 cm/s, $P<0.05$, Figure 7A, B), while the amount of hVSC nuclei were confirmed to be equal in both groups (19.6±6.0% of nuclei in non-fused co-cultures [n=30 pictures with 74±21 nuclei per picture] vs. 21.2±7.0% of nuclei in fused co-cultures [n=42 pictures with 72±23 nuclei per picture, $P>0.05$], $P>0.05$).

Additionally, APD_{90} was significantly shorter and optical action potential morphology was distinctly different by heterocellular fusion (328±56ms vs. 480±79 ms, $P<0.05$, Figure 7C,D). Spatial dispersion of repolarization was also significantly lowered by heterocellular fusion (86±45 ms vs. 156±40 ms in unfused co-cultures, $P<0.05$, Figure 7C, E). Early afterdepolarizations and reentrant arrhythmias were not observed in fused cultures (n = 23), whereas 63% of non-fused cultures (n=8) showed such arrhythmic activity (Figure 7F).

Next, culture excitability was tested by programmed electrical stimulation. Lowest bipolar stimulus voltage to result in 2Hz capture was found to be 2.6±1.4V in fused co-cultures, as opposed to 5.1±2.2V in unfused co-cultures without VSV-G expression (P<0.05, Figure 7G), indicating increased excitability of these cultures by heterocellular fusion. In addition, the maximal capture rate was increased by heterocellular fusion (2.9±0.3 vs. 2.2±0.4 Hz in control cultures, P<0.05, Figure 7H).

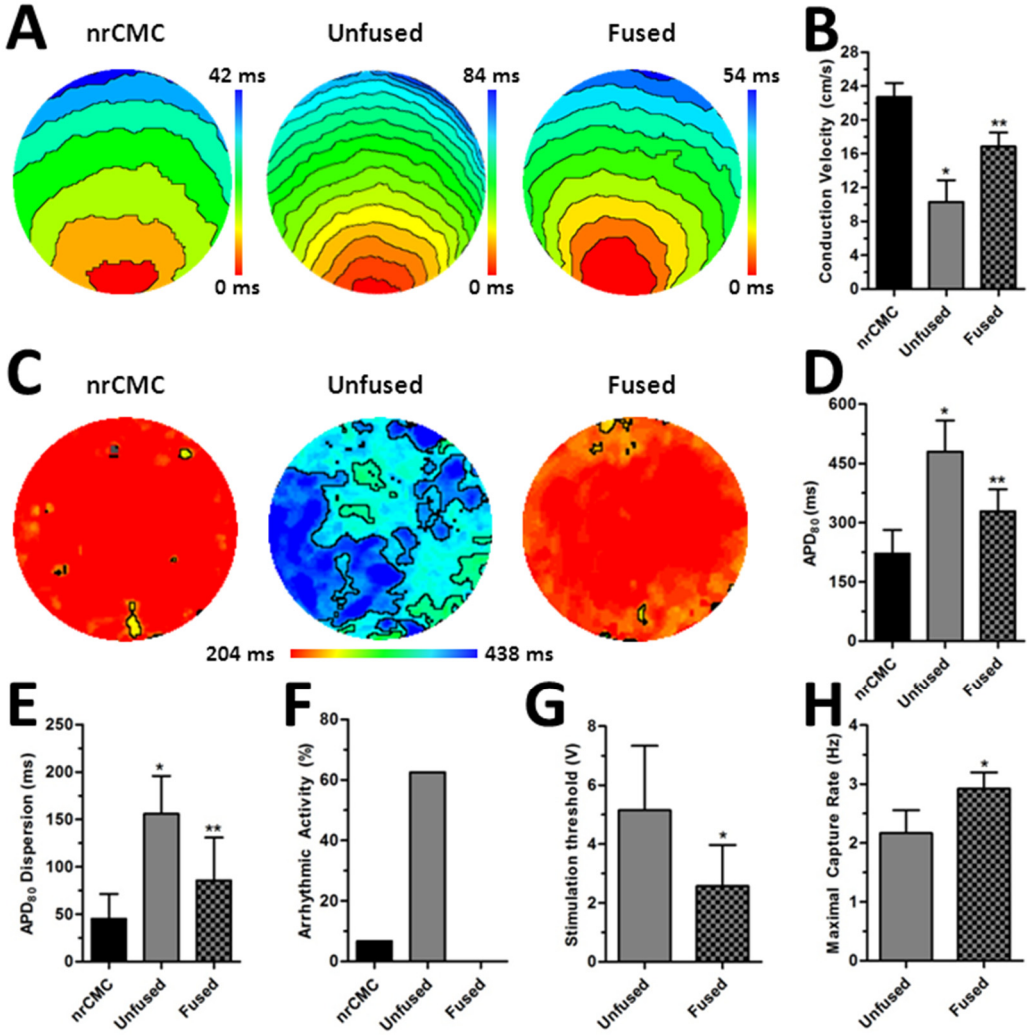

Figure 7. *Forced heterocellular fusion is anti-arrhythmic. (A) Typical activation maps with 6ms isochronal spacing showing (B) increased CV by heterocellular fusion. *:P<0.05 vs. nrCMC and fused co-cultures. **:P<0.05 vs. unfused co-cultures and nrCMCs. (C) Typical APD maps of nrCMC cultures, unfused co-cultures and fused co-cultures. (D) APD was shortened in cultures with heterocellular*

*fusion. *:P<0.05 vs. nrCMC and fused co-cultures. **:P<0.05 vs. unfused co-cultures and nrCMC cultures. (E) APD dispersion was decreased by heterocellular fusion. *:P<0.05 vs. nrCMC cultures and fused co-cultures. **:P<0.05 vs. unfused co-cultures and nrCMC cultures. (F) Arrhythmic activity was ameliorated by heterocellular fusion. (G) The threshold of electrical stimulation to evoke electrical activation was lower in fused co-cultures. *:P<0.05 vs. unfused co-cultures. (H) Maximal capture rate is increased in fused co-cultures. *:P<0.05 vs. unfused co-cultures.*

Discussion

Key findings of this study are (1) heterocellular fusion of hVSCs and nrCMCs is feasible, well-tolerated and preserves cellular structural integrity and (2) can be anti-arrhythmic (3) Moreover, the nrCMC phenotype appears to be dominant in heterokaryons, suggesting heterocellular fusion may serve as a strategy to reprogram fibroblasts towards cardiomyocytes.

Pro-arrhythmic aspects of fibroblasts

Fibrosis, an increase in extracellular matrix deposition and number of fibroblasts in response to cardiac injury, can cause conduction slowing and block, which predispose to potentially lethal cardiac arrhythmias.[13] Apart from the anatomical obstructions provided by the ECM, recent evidence suggests that fibroblasts themselves may be pro-arrhythmic through several mechanisms. Due to the poor gap-junctional coupling capacity of fibroblasts, these cells act as current-sinks with a high intercellular resistance.[14,15] Conduction is hindered even further by their inexcitability, which is paramount for fast conduction and therefore leads to slow, electrotonic conduction.[14] Moreover, fibroblasts can intersperse between CMCs and may therefore obstruct conduction by reducing the gap-junctional communication between CMCs due to physical separation. Therefore, conduction slowing and block by fibroblast interspersion may play an important role in fibrosis-associated arrhythmias. By increasing gap-junctional coupling of fibroblasts, conduction block could be overcome *in vitro*, implying a potentially anti-arrhythmic effect of such an intervention.[16] However, modulating electrical fibroblast integration as an anti-arrhythmic strategy may proof to be more challenging, as fibroblast inexcitability and their depolarized membrane can negatively affect CMC electrophysiology through gap-junctional coupling.[2,15,17,18] Overcoming the inexcitability, depolarized membrane and poor intercellular coupling of fibroblast may represent a robust anti-arrhythmic strategy that is becoming increasingly successful.[19-21] In the current study, a novel alternative solution which consisted of forced cellular fusion of hVSCs with nrCMCs in an attempt to fully electrically integrate these fibroblastic cells into the cardiac syncytium was investigated. By heterocellular fusion, the amount of interspersed fibroblastic cells that were taken from human post-myocardial infarction scars was decreased while the amount of nuclei

remained the same. Thereby, electrical integration of hVSCs would be expected to be maximized as per definition, intercellular resistance is non-existent if cells have been fused together into one hybrid cell. Such forced heterocellular fusion decreases the amount of interspersed, high resistance current-sinks and forced the incorporation of inexcitable cellular membrane of a fibroblast into an excitable membrane of the CMC. Overall, these changes translated to an increased CV in fused co-cultures, indicating that maximizing fibroblast integration by incorporating them within an excitable membrane may represent a novel anti-arrhythmic strategy. Although cellular fusion has been previously used to induce ion channel expression in cells,[22] the current study is, to our knowledge, the first to use such a strategy as an anti-arrhythmic intervention.

Electrophysiological effects of cellular fusion

Although cellular fusion is an established phenomenon in biology, its implications for cardiology have mostly been studied in the context of regenerative medicine and stem cell differentiation while little research has focused on its implications for electrophysiological concepts.[5] In the current study, a recently developed protocol of inducing cellular fusion was utilized, which is termed V-fusion.[10] By overexpressing the Vesicular Stomatitis Virus G-protein in a cell and subsequently lowering the extracellular pH to 6.0 for a brief period, fusion was easily induced in a more efficient manner than older methods of cellular fusion.[10] In addition, V-fusion only causes cellular membrane fusion without causing fusion of intracellular membranes as seen with other types of cellular fusion induction and thereby appears to be a solid, well-tolerated technique to investigate the effects of cellular fusion on electrophysiology.[23] Theoretically, by cellular fusion, intercellular resistance becomes void while cellular capacitance increases as the cellular volume increases with the amount of fused cells. While the increased cellular capacitance may delay phase 0 of the action potential and thereby slow conduction, the decreased intracellular resistance increase CV.[13] Moreover, the altered source-sink relationships caused by increased capacitance through heterocellular fusion may lower the ease by which EADs are propagated. [24] In the current study, cultures with heterocellular fusion showed no EADs, while unfused hVSCs did cause EADs. Apart from altered source-sink relations, the APD shortening that was accomplished by heterocellular fusion may also contribute to the lack of observed EADs. Moreover, overall culture excitability was increased, as electrical stimulus threshold was significantly lowered by heterocellular fusion. Considering the importance of excitability for fast conduction, CV may have been increased by increased excitability in fused co-cultures. Besides the anti-arrhythmic implications of increased excitability, the lower electrical stimulus threshold to evoke electrical activation may therefore also be relevant to cardiac resynchronization therapy, of which the efficacy is hindered by the presence of scar tissue. [25]

Phenotypical dominance of nrCMCs within heterokaryons

Increased excitability of fused co-cultures was likely to be a result of the expression of cardiac ion channels from the nrCMC part of the heterokaryon, as cav1.2 was found to be expressed within the membrane of heterokaryons. Indeed, cellular fusion leads to merging of cellular membranes, and thereby their integrins and ion channels are expressed and functional in the resultant hybrid membrane. [22] In addition, connexin expression was also increased for heterokaryons compared to unfused hVSCs and was equal to connexin expression levels of nrCMCs. This suggests that the degree of electrical integration of heterokaryons into the cardiac syncytium was higher compared to unfused hVSCs. However, if heterokaryons were just a sum of their parts, their expression levels of Cx43 would not be expected to be equal to nrCMC expression levels and therefore, dominance of the nrCMC phenotype is likely. In line with these findings were the findings that expression levels of Cx43, vimentin or Cav1.2 did not linearly correlate with the nuclear composition of the heterokaryon. Phenotypical dominance of nrCMCs within heterokaryons in our study was further supported by the observation that most heterokaryons retained sarcomeric α-actinin expression and were contractile, which is consistent with current literature.[26] Since our purified nrCMC cultures contained a small amount of endogenous fibroblasts, it is likely that the low numbers of heterokaryons that did not express α-actinin contained neonatal rat fibroblasts instead of nrCMCs. Inversely, as vimentin is more dominantly expressed in fibroblasts, the steep decrease in vimentin expression in fused cells compared to non-fused hVSCs is also consistent with dominance of the nrCMC phenotype within heterokaryons. The mechanism of nrCMC dominance is unlikely to be based on a simple “majority-rules” principle, as heterokaryons were often composed of more hVSCs than nrCMCs while retaining cardiac-specific protein expression at levels higher than to be expected for such a principle. Rather, the mechanism of nrCMC dominance within heterokaryons may be more complex. By forcing multiple nuclei inside the same cytoplasm, exposure to normally separated intracellular environments that are rich in celltype specific gene, mRNA, miRNA and protein expression profiles may occur. Such pancellular cross-talk can influence all present nuclei to alter their gene expression profiles[27,28]. Since CMCs can increase their size and protein expression profiles in response to mechanical[29] and humoral stimuli[30] and even changes in ionic currents,[31] the dominance of the nrCMC phenotype may lie in increased transcriptional and translational activity in response to the strong alterations in cellular configuration after heterocellular fusion.

Although hVSCs were outnumbered by nrCMCs (in a 1:4 ratio) in the current study, the average number of human nuclei within a heterokaryon exceeded the more predictable value of 20%, indicating that homocellular hVSC-fusion is favored. This is likely due to VSV-G expression in both cells that allows for easier fusion than in heterocellular fusion.

Despite the relatively high amount of human nuclei within heterokaryons, the apparent phenotypical dominance of CMCs within heterokaryons suggests that heterocellular fusion may even have anti-arrhythmic effects in circumstances where CMCs are outnumbered by fibroblasts. As modifying fibroblasts to resemble or even mimic CMCs is a key focus in current anti-arrhythmic research, heterocellular fusion may represent a novel and promising candidate for more in-depth exploration.

Future Perspectives

Cardiac architecture and intercellular contacts are highly regulated *in vivo* through intercalated discs and anisotropic fiber direction to optimize conduction and contraction. Cellular fusion may interfere with these important aspects of cardiac physiology and may be undesirable as a widespread anti-arrhythmic solution in the 3-dimensional heart. Therefore, heterocellular fusion would be most suited for sites with a high degree of CMC-Fibroblast interactions that are prone to arrhythmias, such as the infarct border zone of which selective genetic modification can yield anti-arrhythmic effects.[32] *In vivo*, extracellular matrix deposition may limit physical contact between CMCs and fibroblasts and thereby limit the potential for fusion to occur. However, such deposition of matrix occurs in later healing phases and adequate timing of fusogenic interventions may circumvent such hindrances. In addition, should the dominance of the CMC phenotype prevail *in vivo* as well, reduced extracellular matrix deposition may occur by heterocellular fusion. By increasing tissue excitability, heterocellular fusion may have implications for cardiac resynchronization therapy as lower voltages could be used for electrical stimulation. Although such perspectives must remain speculative for the time being, the strong anti-arrhythmic effects of heterocellular fusion demonstrated in this proof-of-concept study provide strong incentive to explore the feasibility of forced heterocellular fusion as an anti-arrhythmic intervention.

Conclusion

Forced heterocellular fusion of hVSCs with nrCMCs is feasible and well-tolerated and produces a hybrid, contractile cell type. Moreover, forcing cellular fusion in hVSC-nrCMC co-cultures is anti-arrhythmic. Moreover, nrCMCs appear to be dominant within the heterokaryon which indicates that heterocellular fusion may represent a novel concept of anti-arrhythmic reprogramming of fibroblasts.

Study limitations

In the current study, neonatal rat cardiomyocytes were utilized due to their availability and ability to remain in culture for extended periods of time, although use of human adult cardiomyocytes would bear more clinical relevance. Nevertheless, using a 2d-model with these cells has proven to be relevant for studying key electrophysiological processes in the

heart. The use of 2 species in our fusion experiments was necessary to distinguish between human and rat nuclei and determine heterokaryon compositions. Despite the similarities between rat and human cells, interspecies differences and effects that depend on these differences are unlikely but possible. Although staining showed that ion channel and connexin expression did not linearly depend on nuclear composition of the heterokaryon, a technique to correct functional ion channel measurements for the heterokaryon nuclear composition is currently unknown due to technical limitations. As a result, despite the implied likelihood of non-linear functional effects of heterocellular fusion on ionic currents, such “dose-dependent” effects cannot be determined at a functional level in the current study, and need to be investigated in a dedicated future study with novel technical refinements.

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