Forced Alignment of Mesenchymal Stem Cells Undergoing Cardiomyogenic Differentiation Affects Functional Integration With Cardiomyocyte Cultures

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Abstract—Alignment of cardiomyocytes (CMCs) contributes to the anisotropic (direction-related) tissue structure of the heart, thereby facilitating efficient electrical and mechanical activation of the ventricles. This study aimed to investigate the effects of forced alignment of stem cells during cardiomyogenic differentiation on their functional integration with CMC cultures. Labeled neonatal rat (nr) mesenchymal stem cells (nrMSCs) were allowed to differentiate into functional heart muscle cells in different cell-alignment patterns during 10 days of coculture with nrCMCs. Development of functional cellular properties was assessed by measuring impulse transmission across these stem cells between 2 adjacent nrCMC fields, cultured onto microelectrode arrays and previously separated by a laser-dissected channel (230±10 μm) for nrMSC transplantation. Coatings in these channels were microabraded in a direction (1) parallel or (2) perpendicular to the channel or were (3) left unabraded to establish different cell patterns. Application of cells onto microabraded coatings resulted in anisotropic cell alignment within the channel. Application on unabraded coatings resulted in isotropic (random) alignment. After coculture, conduction across seeded nrMSCs occurred from day 1 (perpendicular and isotropic) or day 6 (parallel) onward. Conduction velocity across nrMSCs at day 10 was highest in the perpendicular (11±0.9 cm/sec; n=12), intermediate in the isotropic (7.1±1 cm/sec; n=11) and lowest in the parallel configuration (4.9±1 cm/sec; n=11) (P<0.01). nrCMCs and fibroblasts served as positive and negative control, respectively. Also, immunocytochemical analysis showed alignment-dependent increases in connexin 43 expression. In conclusion, forced alignment of nrMSCs undergoing cardiomyogenic differentiation affects the time course and degree of functional integration with surrounding cardiac tissue. (Circ Res. 2008;103:167-176.)

Key Words: stem cells  ■  alignment  ■  integration  ■  electrophysiology  ■  cell culture

The developing heart is characterized by increases in the number and size of cells resulting from hyperplasia and hypertrophy.1-2 During this process, the initially round-shaped cardiomyocytes (CMCs) become elongated through unidirectional growth and align in a specific direction, thereby defining a long and short cellular axis.3 Later in development, intercalated disc components (including gap junction proteins), initially distributed more or less uniformly over the surface of the cells, cluster at the polar ends of the cells.4,5 Consequently, the elongated terrace-shaped cells are coupled end-to-end to surrounding cells and organized in unit bundles. This anisotropic tissue architecture has implications for the electrical activation of the cardiac muscle. Conduction of the electrical impulse parallel to the myocardial fiber axis is three times faster than perpendicular to the fiber axis, indicating anisotropic conduction.6 In the intact heart, the resulting inhomogeneity in conduction is circumvented by a 120 degrees rotation of the fiber axis from epicardium to endocardium.

As a consequence, the spread of activation through the ventricular muscle is more or less homogeneous, coordinated, and fast.7,8 Any disruption of this architecture, for example, after myocardial infarction, may cause conduction abnormalities associated with diminished pump function and increased risk of arrhythmias.9

Recently, stem cell therapy has been introduced as a treatment option to improve left ventricular function after myocardial infarction.10 Ideally, to be successful, stem cells should not only differentiate into CMCs but also engraft and align with the surrounding anisotropic tissue. This latter process can be referred to as spatial integration. As a result, these de novo CMCs will be an integral part of the 3D myocardial architecture, thereby contributing to cardiac impulse conduction. Until now, it has been unknown how transplanted stem cells integrate with the surrounding myocardial cells and to what extent alignment of these cells may influence electrical conduction. In other words, spatial inte-
igation of stem cell-derived CMCs may contribute to restoration of tissue structure and conduction or, in contrast, result in increased structural and electrical inhomogeneity. In theory, each cell type that is coupled electrically, excitable, and aligned can acquire anisotropic properties as in this situation the resistance in the transverse direction is higher than in the longitudinal direction. In case of stem cells, this is of special interest as they may acquire anisotropic properties during cardiomyogenic differentiation after they have been transplanted into the anisotropic myocardium, thereby potentially improving their functional integration with the surrounding myocardium. Of note, functional integration of such transplanted cells is essential for cell therapy to be safe and effective.11

In the present study, we used a standardized 2D in vitro coculture model with a growth-directing substrate12,13 to investigate the effects of cell alignment on functional integration and electrical conduction across neonatal rat mesenchymal stem cells (nrMSCs) undergoing cardiomyogenic differentiation. The process of differentiation occurred in a cardiac syncytium of neonatal rat (nr)CMCs, thereby allowing the study of functional integration.

Materials and Methods

Animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the NIH.

A detailed description of harvesting and culturing of nrCMCs, nrMSCs, and cardiac fibroblasts (nrCFBs), as well as the characterization of the nrMSCs, can be found in the online data supplement at http://circres.ahajournals.org.

Assessment of Functional Cardiomyogenesis

Differentiation of nrMSCs was assessed by a combination of immunofluorescence microscopy and electrophysiological measurements in isotropic cocultures of nrCMCs and enhanced green fluorescent protein (eGFP)-labeled nrMSCs grown on glass coverslips during the course of 10 days. The assessment of cardiomyogenesis by immunofluorescence and intracellular measurements is described in the online data supplement.

Microelectrode High-Density Mapping

Simultaneous microelectrode high-density mapping of cultured nrCMCs and nrMSCs was performed using microelectrode arrays (MEAs) (number of titanium nitride electrodes: 60; interelectrode distance: 200 μm; electrode diameter: 30 μm) and associated data acquisition system (sampling rate 5 kHz/channel, Multi Channel Systems, Reutlingen, Germany). Further descriptions can be found in the online data supplement.

Induction of Conduction Block and Anisotropic Cell Alignment

Activation maps of nrCMCs were generated 2 days after culture to confirm the presence of a synchronously beating monolayer. Conduction block was generated using a P.A.L.M. microlaser system (Micro laser Technologies GmbH, Bernried, Germany).14 Briefly, 2 programmed linear laser dissections were made, separated by 225 μm, crossing the entire diameter of the monolayer in the coated MEA culture dish. This resulting in a detached strip of monolayer between the 2 laser dissection lines, which was removed from the culture, creating a clean acellular channel electrically separating the 2 nrCMC fields. Subsequently, the uncovered coatings in the acellular channels were given a microgroove pattern by microabrasion14 with a soft microbrush (bristle diameter: 30 μm diameter) using a micromanipulator (Seirz, Göttingen, Germany) and light microscope (×40 magnification), either in a direction (1) parallel or (2) perpendicular to the acellular channel. (3) Another group consisted of MEA culture dishes with an acellular channel that was not microabraded.

After confirming the presence of a conduction block between the 2 nrCMC fields, (1) 5×10⁴ CM-Dil-labeled nrCMCs, (2) 5×10⁴ eGFP-labeled nrMSCs, or (3) 5×10⁴ eGFP-labeled nrCFBs were applied in a channel-crossing pattern in each of the 3 groups. This was achieved by gently adding the cells onto the coating in between the CMC fields, using a pipette fixed to a micromanipulator in combination with a light microscope (×20 magnification). After 24 hours, the culture medium was refreshed to remove nonattached cells, as well as 1 hour before and after measurements. During the following 10 days, the electrical conduction (impulse transmission) across seeded cells was assessed daily.

The 2 separated and asynchronously beating nrCMC fields were considered electrically coupled on application of cells, if the timing of the electrograms of the 2 nrCMC fields correlated consistently with each other for 30 consecutive local activation times recorded at both fields, while stimulating 1 nrCMC field.

In an additional series of experiments, a mixture of 5×10⁴ nrCMCs and nrCFBs (20%:80%) was applied to the channel and subjected to electrophysiological measurements 24 hour after seeding and serving as positive control for the results obtained in the nrMSC group at day 10.

Statistics

Statistical analysis was performed using SPSS 11.0 for Windows (SPSS Inc, Chicago, Ill). Data were compared with Student t test or ANOVA test with Bonferroni correction for multiple comparisons and expressed as means±SD for a given number (n) of observations. Probability values <0.05 were considered statistically significant.

Results

Characterization of Bone Marrow–Derived nrMSCs

Analysis of In Vitro Adipogenic and Osteogenic Differentiation Potential

The nrMSCs (Figure 1A1) were assessed for their multipotency by investigating their adipogenic and osteogenic differentiation potential. After incubation in appropriate differentiation media, nrMSCs readily differentiated into adipocytes and osteoblasts, as determined by formation of lipid vacuoles and calcium deposits, respectively (Figure 1A2 and 1A3).

Analysis of Surface Marker and Connexin Expression

A very large fraction of the nrMSCs (p2) expressed the hematopoietic marker CD34 (1.5%). CD44 (32.8%), CD45 (10.2%), and CD106 (10.3%) were expressed on their surface, whereas almost none of them stained positive for the mesenchymal markers CD29 (97.3%) and CD90 (96.2%) at day 10. Differentiation by investigating their adipogenic and osteogenic differentiation potential.

At day 10, positive staining for Cx43, Cx40, and Cx45 had increased significantly (P<0.01, Figure 2E), without a no-
At day 10 after cell seeding, only Cx43 and Cx45 staining had significantly increased ($P < 0.01$) as compared to day 3 of culture, but distribution patterns did not change over time. In contrast, no increase in Cx40 staining was observed in or between cells at day 10 of culture (Figure 2D1 through 2D3).

Assessment of Cardiomyogenic Differentiation

Immunocytochemistry

At day 3 of coculture, a fraction of the eGFP-labeled nrMSCs stained positive for sarcomeric $\alpha$-actinin and cardiac troponin I in a diffuse and speckled staining pattern (Figure 3A2 and 3A4). However, a larger fraction of nrMSCs stained negative for both markers (Figure 3A1 and 3A3). Cross-striation in nrMSCs was first observed at day 6, whereas at day 10, $\approx 17\%$ of the eGFP-labeled nrMSCs showed typical cardiac cross-striated patterns of sarcomeric $\alpha$-actinin (Figure 3D1 through 3D3) and cardiac troponin I (Figure 3D4 through 3D6). Most of the nrMSCs displaying cross-striation were adjacent to native CMCs. Importantly, none of the nrMSCs having cross-striation of contractile proteins (>60 cells analyzed per sarcomeric protein type) were multinuclear, making cell fusion of eGFP-labeled cells with nrCMCs unlikely. Total positive staining for sarcomeric $\alpha$-actinin and cardiac troponin I in eGFP-labeled cells increased significantly from $34 \pm 5\%$ and $30 \pm 6\%$ at day 3 to $63 \pm 3\%$ and $60 \pm 4\%$ at day 10 (now including $17.1 \pm 3\%$ and $16.3 \pm 4\%$ of cells that show positive staining with cross-striation), respectively ($P < 0.001$) (Figure 3B). Furthermore, sarcomere length (ie, distance between 2 Z-lines) in differentiated nrMSCs at day 10 was comparable to that in native nrCMCs (Figure 3C1 and 3C2). Samples having irregularities in sarcomere structures (<10% of samples) were excluded.

Electrophysiological Measurements After Uncoupling

Patch-clamp recordings were obtained from nrCMCs and eGFP-labeled nrMSCs at day 3 and day 10 of coculture after electrical isolation with the gap junction uncoupler 2-aminoethoxydiphenyl borate (2-APB). On 2-APB treatment, the synchronously beating monolayer disintegrated into asynchronously beating cells, and input resistance increased from 20 to 120 M$\Omega$ ($n = 15$) to 0.9 to 1.2 G$\Omega$ (18 other cells). These increases in input resistances were considered to reflect electrical uncoupling of the patched cell from the surrounding cells, thereby reaching the approximate seal resistance. After electrical uncoupling at day 3, eGFP-labeled nrMSCs ($n = 9$) had maximal diastolic potentials of $-16 \pm 5$ mV and showed no spontaneous action potentials (APs). In contrast, uncoupled nrCMCs ($n = 12$) had maximal diastolic potentials of $-69 \pm 8$ mV in the presence of spontaneous APs. Interestingly, after 10 days of coculture and incubation with 2-APB, a considerable fraction of nrMSC-derived nrCMCs ($\approx 16\%$; $n = 9$) was found to be beating independently from surrounding nrCMCs (as judged by timing and frequency of beating), while showing AP characteristics (maximal diastolic potential: $-63 \pm 4$ mV) comparable to native nrCMCs ($n = 10$) (Figure 4A1 and 4A2 and 4B1 through 4B4). The remaining

Figure 1. Characterization of nrMSCs. A1, Bright field image of cultured nrMSCs. A2, Presence of oil red O–stained fat vacuoles after adipogenic differentiation. A3, Calcium accumulation was visualized by alizarine red S staining after osteogenic differentiation. B, Flow cytometric analysis of nrMSCs showed abundant surface expression of CD29 and CD90 but hardly any expression of the hematopoietic marker CD34. Furthermore, CD44, CD45, and CD106 were expressed at low or medium levels. Flow cytometric analyses with isotype-matched control antibodies are included to determine background fluorescence levels (thin black line).

Figure 2B1 through 2B3. At day 10 after cell seeding, only Cx43 and Cx45 staining had significantly increased ($P < 0.01$) as compared to day 3 of culture, but distribution patterns did not change over time. In contrast, no increase in Cx40 staining was observed in or between cells at day 10 of culture (Figure 2D1 through 2D3).
Microabrasion of the coating, either parallel or perpendicular nMSC resulted in a confluent, isotropic cell layer (Figure 5A1). Cultures were included for further experiments. As a consequence, only 40% of the mapping. Furthermore, damaged and/or detached nrCMC determined by light microscopy and electrophysiological and functional homogeneity were included in this study, as Only confluent monolayers with a high degree of structural

to the channel, resulted in alignment of each cell type according to the direction of abrasion (Figure 5B1 and 5C1). nMSCs, as other applied cells, appeared elongated with an average length/width ratio of 4:1 and maintained their shape throughout follow-up (Figure 5D1 and 5D2). In contrast, cells applied onto unabraded coatings had a variable length/width ratio (Figure 5D3) but never reaching 4:1 of anisotropically aligned cells.

Effect of Cell Alignment on Conduction Velocity and Functional Integration

After creating an acellular channel (230±10 μm) in the MEA culture dish, 2 asynchronously beating nrCMC fields were present, proving the presence of a conduction block. Conduction velocity (CV) across the 2 isotropic nrCMC fields was 21.3±2.3 cm/sec at day 1 and increased slightly to 22.7±2.6 cm/sec at day 10.

Isotropic Cell Alignment

Application of nrCMCs onto unabraded coatings in the channels resulted in conduction between the 2 nrCMC fields within 1 day. CV across nrCMCs in the channel was 19.7±0.7 cm/sec at day 1 and 20.7±1 cm/sec at day 10 (n=12; P=NS) (Figure 5A2), which was comparable to the CV values measured across neighboring nrCMC fields (Figure 6A1). Application of nrMSCs in the channel also restored conduction between both nrCMC fields, but CV was significantly lower: 1.7±0.7 cm/sec (P<0.01). CV across these isotropically aligned nrMSCs increased to 7.1±1 cm/sec at day 10 (n=11; P<0.01) (Figures 5A2 and 6A1). Electrical conduction between the nrCMC fields was also restored after application of nrCFBs; however, CV across these cells was 1.8±0.8 cm/sec at day 1 and remained stable until day 10 (n=12; P=NS and Figure 6A1), being significantly lower than the CV across nrMSCs at day 10 (P<0.01) (Figure 5A2).

Anisotropic Cell Alignment: Parallel Versus Perpendicular Cell Alignment

Application of nrCMCs onto coatings abraded in a direction parallel to the channel resulted, after 1 day, in electrical recoupling of the 2 nrCMC fields, which was associated with a CV of 13.5±0.9 cm/sec across the nrCMC-filled channel and persisted during follow-up (CV: 14.1±1 cm/sec; n=10; P=NS) (Figures 5B2 and 6B1). Application of nrCMCs onto coatings abraded in a direction perpendicular to the channel, however, resulted in a CV of 26.0±1.1 cm/sec (P<0.01) across the channel, which also persisted during the follow-up until day 10 (CV: 26.8±0.9 cm/sec; n=11; P=NS) (Figures 5C2 and 6C1).

In contrast, application of nrMSCs onto coatings abraded in a direction parallel to the channel failed to restore electrical conduction between the 2 nrCMC fields up to day 7. However, at day 7, restoration had occurred and was associated with a CV of 4.9±1 cm/sec at day 10 (n=11; P<0.01) (Figures 5B2 and 6B1). Application of nrMSCs onto perpendicularly abraded coatings restored electrical conduction between the 2 nrCMC fields from day 1 onward. CV increased from 4.3±1 cm/sec at day 1 to 11±0.9 cm/sec at day 10 (n=12; P<0.01) (Figures 5C2 and 6C1), thereby reaching the highest CV in the nrMSC group in the different configurations.

nrMSCs (n=47) were nonbeating and had a maximal diastolic potential of −19±4 mV in the absence of APs (Figure 4A2).

Cell Alignment After Microabrasion

Only confluent monolayers with a high degree of structural and functional homogeneity were included in this study, as determined by light microscopy and electrophysiological mapping. Furthermore, damaged and/or detached nrCMC fields, attributable to laser dissection or microabrasion, were excluded from the study. As a consequence, only 40% of the cultures were included for further experiments. Application of nrCMCs, nrMSCs, or nrCFBs onto the uncovered fibronectin–gelatin coating in the acellular channel resulted in a confluent, isotropic cell layer (Figure 5A1). Microabrasion of the coating, either parallel or perpendicular

**Figure 2.** Immunocytochemical analysis of Cx staining in isotropic cocultures of nrMSCs and nrCMCs at days 3 and 10 of culture. Staining for Cx43 and Cx45 increased significantly over time in nrMSCs (A1, A3, C1, and C3), as well as in nrMSCs adjacent to nrCMCs (B1, B3, D1, and D3). Positive staining for Cx40 increased only in MSCs during follow-up (A2 and C2). Quantitative analysis (E) was based on 36 random samples taken from 12 adjacent nrMSC-nrMSC and nrCMC-nrMSC cell pairs on 3 different coverslips at each time point. White arrows pinpoint the presence of Cxs at the site of gap junctions. *P<0.01 vs nrMSCs at day 3, **P<0.01 vs nrMSC-nrCMC pairs at day 3.
This indicates that alignment of nrMSCs undergoing cardiomyogenic differentiation influences the degree of functional integration with respect to CV across adjacent cardiac tissue.

Application of nrCFBs onto the parallel abraded coatings did not result in electrical conduction between the 2 nrCMC fields during 10 days of culture (n=10; P<0.0001) (Figures 5B2 and 6B1). However, after application of nrCFBs in
perpendicular abraded coatings, electrical conduction was restored at day 1, associated with a CV across these cells of $3.5 \pm 0.8$ cm/sec at day 1 and $4.3 \pm 1$ cm/sec at day 10 ($n=10$; $P=\text{NS}$) (Figures 5C2 and 6C1).

Interestingly, quantitative analysis of Cx43 in different cells, configurations, and locations at day 10 of culture showed significant differences, revealing that the highest Cx43 expression was found in the group of parallel alignment (Figure 6, corresponding A2, B2, and C2).

The degree of cell alignment and confluence did not change significantly over time, as was shown by quantification and spatial measurements using (immuno)fluorescence microscopy (data not shown).

Conduction Velocity Across Control Cultures of Cardiomyocyte/Fibroblast Mixtures

To mimic the composition of the cell population in the channel at day 10, which consisted of nrMSCs-derived CMCs and undifferentiated nrMSCs (~18% and ~82%, respectively), mixtures of native nrCMCs and nrCFBs (20% and 80%) were applied in all 3 different configurations ($n=8$, 8 and 9). After application of these mixtures onto the coatings, CVs determined at day 1 were comparable to CVs across eGFP-labeled nrMSCs at day 10 of culture ($n=11$, 10 and 10) (Figure 7). This suggests that the increase in CV across eGFP-labeled MSCs that was observed depends on the functional cardiomyogenic differentiation of nrMSCs.

Figure 4. Whole-cell patch-clamp recordings from nrCMCs and eGFP-labeled nrMSCs after 10 days of coculture and gap junction uncoupling. Stable and spontaneous APs were recorded in (A1) nrCMCs ($n=10$), as well as in (A2, left) differentiated eGFP-labeled cells ($n=9$). In contrast, undifferentiated, nonbeating nrMSCs had resting membrane potentials of $-19 \pm 4$ mV, in the absence of APs (A2, right; $n=47$). AP characteristics were comparable to each other, as shown by maximum diastolic potential (MDP) (B1); AP amplitude (APA) (B2); AP duration until 50% repolarization (APD_{50}) (B3); and maximal rate of depolarization (dV/dt_{max}) (B4).

Discussion

The present study shows that alignment of transplanted nrMSCs undergoing cardiomyogenic differentiation affects the time course and degree of functional integration with cultured nrCMC tissue.

Functional Differentiation of nrMSCs

In this study, a significant fraction of nrMSCs differentiated into functional CMCs after coculture with nrCMCs. In additional coculture experiments, differentiation percentages of nrMSCs were assessed at day 14, showing ~18% differentiation (compared to ~17% differentiation at day 10), which may indicate that maximal differentiation percentages were reached under our culture conditions. Previously, Nishiyama et al also showed the cardiomyogenic differentiation potential of premature MSCs after coculture with CMCs.18 The results of our study confirm these results using nrMSCs and nrCMCs. In addition, Jiang et al demonstrated that MSCs from young rats differentiated into cardiac-like cells after transplantation in the infarcted rat myocardium, whereas MSCs from old rats did not.19

In the present study, differentiation of nrMSCs into functional CMCs was also demonstrated by intracellular (patch-clamp) membrane potential recordings after uncoupling. One might argue that the 2-APB–induced uncoupling in the patch-clamp experiments may be incomplete and that 180 µmol/L 2-APB may have affected plasma membrane ion channels, thereby affecting excitability of the nrCMCs and differentiated nrMSCs. However, the observed desynchronized beating and high whole-cell input resistance (~1 GΩ) in the presence of 2-APB indicated preserved single-cell excitability and strong uncoupling. These findings, together with the presence of CMC-like APs in the uncoupled differentiated nrMSCs but not in uncoupled undifferentiated nrMSCs, are indicative for functional differentiation of at least a fraction of the nrMSCs.

Alignment of Stem Cell–Derived Cardiomyocytes and Electrical Conduction

Several studies have shown anisotropic conduction across aligned CMCs in vitro. The longitudinal and transverse CVs measured across nrCMCs in this study are in line with the results of previous studies using the same source,13 although some studies have reported higher CVs.15,20 These differences in CV might have been caused by the presence of different numbers of cardiac fibroblasts in the CMC culture or by the
methods used to produce anisotropic cell cultures.\textsuperscript{13} However, in our study, we also applied other cells than CMCs to the microabraded coatings, and these also appeared elongated with oval shaped nuclei and a certain level of Cx43 uniformly distributed along cell–cell contacts. Anisotropic alignment of cells in our model most likely created a low-resistance conduction pathway in longitudinal direction, where fewer (high-resistance) cell borders have to be crossed, thereby favoring conduction in this direction. These phenomena were not restricted to a specific cell type. However, after culture

**Figure 5.** The effect of cell alignment on the development of conduction across the cell strip displaying a random (A1), parallel (B1), or perpendicular configuration (C1). D1, Cells applied on microabraded coatings showed typical alignment throughout follow-up. D2, After alignment, nrMScs appeared elongated with oval shaped nuclei (average length $L$/width $W$ ratio of 4:1) and displayed Cx43 staining uniformly distributed along cell–cell contacts. D3, In contrast, application of cells onto unabraded coatings resulted in random cell alignment, with a variable length/width ratio (but always lower than the 4:1), which was maintained until day 10. A2, B2, and C2, Two-factor mixed ANOVA test (Bonferroni-corrected); \*P < 0.05 vs CV at day 10 in random configuration; †P < 0.01 vs CV at day 10 in parallel configuration; ‡P < 0.01 vs CV at day 10 in perpendicular configuration. One-way repeated-measures ANOVA (Bonferroni-corrected).
and cardiomyogenic differentiation of nrMSCs the anisotropic electrical properties further developed and functional integration with adjacent CMC fields improved. The anisotropic ratio (perpendicular CV versus parallel CV) was comparable between nrCMCs and nrMSCs at day 10 of culture; however, only CV across nrMSCs increased over time.

Distribution patterns of Cx43 did not appear to be of major influence on conduction, which has been confirmed by other studies.\(^1^5\) The uniform distribution of Cx43 in nrCMCs, reported in the present and other studies, is typical for neonatal CMCs. Importantly, cells aligned parallel to the channel, thereby having adjacent, beating cardiac tissue perpendicular to their cell axis, were associated with the highest increase in Cx43 expression levels. A previous study reporting on neonatal CMCs subjected to anisotropic stretch showed a higher increase in Cx43 expression in CMCs stretched perpendicular to their cell axis than when CMCs were stretched in a parallel direction.\(^2^2\) In our model, the cells in the channel seem to be influenced by stretch originating from the adjacent CMC fields, thereby indicating that alignment per se is not only affecting functional integration but that it also has an effect on gap junction regulation. In fact, our model could allow the study of stretch-related protein expression levels, as an alternative to mechanically induced pulsatile stretch, which also was shown to significantly increase Cx43 expression in nrCMCs.\(^2^3\) In addition, cardiomyogenic differentiation of nrMSCs may lead to increased Cx expression. Also, long-term culturing of MSCs itself leads to upregulation of Cx expression.\(^2^4\) However, the relative con-

**Figure 6.** A1, B1, and C1, Extracellular electrograms derived from 10-day cultures of nrCMCs, nrCFBs, or nrMSCs cultured in different configurations and from different locations (white background). In gray, electrograms of adjacent CMC fields are shown and referred to as outer-CMCs. A2, B2, C2, Cx43 expression was quantified in all 3 cell types and compared to expression in adjacent CMC fields (hatched bars for outer-CMCs, set to 100%). Cx43 expression was also quantified and compared between all 3 cell types in each configuration. *P<0.05 vs Cx43 expression of outer-CMCs; #P<0.05 vs Cx43 expression in corresponding cells aligned parallel to the channel.

**Figure 7.** Positive control for cardiomyogenesis-related increase in CV across nrMSCs. CVs across nrMSCs at day 10, cultured in 1 of the 3 configurations (n=11, 10, and 10) were compared to CVs across a mixture of 20% nrCMCs and 80% nrCFBs at day 1 (n=8, 8, and 9).
tions of each of these phenomena to the improvement of AP transmission by increased gap junctional coupling warrants further study.

In the present study, nrMSCs in all 3 cell orientation groups showed a time-dependent increase in CV, which correlated with differentiation of nrMSCs, although the maximum CV differed among the groups. Comparison of the CV values of the transplanted nrMSCs with CV values of nrCMCs at different time points revealed the effect of stem cell alignment on functional integration with cardiac tissue. This is best illustrated by the finding that nonexcitable nrMSCs aligned parallel to the channel were unable to conduct the electrical current across the channel for up to 6 days. However, after cardiomyogenic differentiation of these cells, electrical conduction across the channel was established. Most likely, in this situation passive conduction across undifferentiated nrMSCs is supported by active propagation across nrMSC-derived CMCs, resulting in AP transmission across the channel and subsequent activation of the distal CMC field. Thus, the alignment of stem cells not only influences the degree of functional integration, as reflected by CV values, but also the time course of functional integration. Whether alignment also influenced the degree of cardiomyogenic differentiation in itself is intriguing. The minor differences between CVs across mixtures of native nrCMCs and nrCFBs and CVs across differentiated nrMSCs indicate that alignment per se is a major determinant of functional integration. However, the increased expression of Cx43 in the nrMSCs aligned to the channel could suggest that alignment has some effect on cardiomyogenic differentiation, but this increase may also be attributable to the factors mentioned above. Future studies will be necessary to investigate the role of cell alignment in cardiomyogenic differentiation and subsequent effects on structural and electrical integration.

Possible Therapeutic Implications

To our knowledge, no study of stem cell transplantation has yet reported on the effects of cell alignment on electrical conduction, functional integration and the therapeutic implications. Furuta et al have used transplanted sheets of nrCMCs onto the epicardial surface of the heart to improve cardiac function and showed anisotropic electrical conduction across the cell sheets.23 Interestingly, these cells appeared to orientate themselves spontaneously in line with the fiber axis of native cells. However, it is unknown whether and how stem cells align after transplantation into the myocardium of the infarcted heart and to what extent alignment of these cells is influencing conduction and contraction. Of note, alignment of resident ventricular CMCs has major influence on electrical and mechanical activation of the heart,26 but the cellular assessment in vivo is technically challenging. The present study introduces alignment of transplanted stem cells as a novel determinant of functional integration, and although conducted ex vivo, the study may have important implications for future cardiac cell therapy. Presumably, cell alignment will become even more important in the near future, because higher engraftment rates of transplanted cells will be achieved using novel application techniques. For example, misalignment of transplanted cells with respect to the native cardiac architecture might result in increased electrical heterogeneity, potentially leading to arrhythmias, as well as dyssynchronous contraction, leading to decreases in cardiac output.9 On the other hand, enforcing transplanted cells to adapt to the native tissue architecture might contribute to improved therapeutic efficiency and safety. In this view, an important and promising role is reserved for tissue engineering, in which the transplanted cells can be aligned with the use of scaffolds.27,28

Study Limitations

Ideally, the nrCMC fields adjacent to the channel should be made anisotropic to mimic cardiac tissue architecture. However, this was not possible with the techniques described in the study. Although the spatial resolution of the extracellular mapping experiments is acceptable to allow standardized measurements, the relatively low spatial resolution may have resulted in an underestimation of actual CVs. In the present study, a growth-directing substrate was created by microabrasion and although effective and reproducible, this procedure is time-consuming and yields only a limited number of cultures suitable for further study. Nevertheless, our study provides the first evidence that alignment of stem cells undergoing cardiomyogenic differentiation has significant impact on functional integration of these cells with cardiac tissue.

Conclusions

Forced alignment of nrMSCs undergoing cardiomyogenic differentiation affects the time course and degree of functional integration with surrounding host cardiac tissue. This study introduces cell alignment as an important determinant of functional integration of transplanted cells, which may contribute to the improvement of therapeutic outcome and reduction of potential hazards. Further study is needed to determine the full biological, biophysical, and therapeutic relevance of cell alignment in functional integration of transplanted cells with host myocardium.

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