Electrophysiological Maturation and Integration of Murine Fetal Cardiomyocytes After Transplantation

Marcel Halbach, Kurt Pfannkuche, Frank Pillekamp, Agnieszka Ziomka, Tobias Hannes, Michael Reppel, Juergen Hescheler, Jochen Muller-Ehmsen

Abstract—In the present study, we investigated the electrophysiological maturation and integration of immature cardiomyocytes after transplantation; maturation and integration are essential to achieve the cardiac regeneration. Murine fetal cardiomyocytes (FCMs) (d12.5-d15.5) expressing enhanced green fluorescent protein under the control of the α-actin promoter were injected into cryoinjured areas and adjacent myocardium of cryoinjured mouse ventricles. Viable short axis tissue slices (thickness, 150 μm) of the ventricles were prepared 5 to 6 days after transplantation. Glass microelectrodes were used for measurements of action potentials in transplanted FCMs and host cardiomyocytes within the slices. Stimulation at frequencies of up to 10 Hz was performed via a unipolar electrode placed in viable host tissue. Transplanted FCMs could be distinguished clearly from host tissue by their green fluorescence and their electrophysiological properties: maximal upstroke velocity (Vmax) was significantly lower and action potential duration at 50% repolarization (APD50) was significantly longer compared with values of adult cardiomyocytes. Transplanted FCMs surrounded by cryoinjured tissue showed synchronous electrical and contractile activity, which was in no case synchronous with host tissue. Vmax and APD50 of these nonintegrated cells matched values of dissociated adult cardiomyocytes. In contrast, 82% of transplanted FCMs surrounded by viable host tissue were electrically integrated; i.e., electrical and contractile activity was synchronous with host tissue and these cells had more mature action potential parameters (significantly higher Vmax and shorter APD50) compared with nonintegrated FCMs. In conclusion, electrophysiological maturation and integration of transplanted FCMs depend on an embedment in viable host myocardium. FCMs surrounded by cryoinjured tissue maintain physiological but immature AP properties. (Circ Res. 2007;101:484-492.)

Key Words: cardiac electrophysiology [cardiomyoplasty] [coupling] [heart slices] [maturation]

Acutely myocardial infarction and chronic heart failure are among the most frequent causes of morbidity and mortality in Western countries. In the course of physiological repairation processes, cardiomyocytes lost because of myocardial infarction are replaced by scar tissue, leading to an impaired cardiac function and remodeling of the remaining myocardium. Despite advances in the conventional therapy, this loss of functional myocardium remains irreversible. Thus, there is a strong need for a novel therapeutic approach aiming at a regeneration of cardiomyocytes: cardiac cell therapy.

Cardiomyocytes derived from embryonic stem cells (ESC-CMs) are promising candidates for cell therapy via an exogenic replacement of lost cardiomyocytes, because their cardiac phenotype, including cardiac specific protein expression as well as typical cardiac electrophysiological properties, is undoubted. Clinical studies using ESC-CMs have not been performed, because, first, techniques enabling the production and selection of sufficient cell numbers must be developed and potential risks and immunological issues must be investigated in animal studies. Besides studies in rodent models, ESC-CMs have been injected in infarcted sheep hearts, leading to a significant improvement of the left ventricular ejection fraction as compared with sham-operated animals.

The capability of ESC-CMs to form gap junctions and to couple electrically to cardiomyocytes of another origin has been demonstrated by in vitro coculture studies. Because cardiac tissue structure and microenvironment cannot be simulated completely using dissociated cells in vitro, coculture models may not necessarily be fully representative for the in vivo situation. In an animal model of atrioventricular block, ESC-CMs injected into the left ventricular wall constituted ectopic pacemakers, i.e., integrated electrically, at the sites of injection, which was demonstrated by 2D mapping of excitation spread. However, there is no information about the functional integration of ESC-CMs transplanted in-
farcted or healthy hearts with a sinus rhythm and about changes of the electrophysiological properties of ESC-CMs after transplantation.

Using dissociated murine fetal cardiomyocytes (FCMs) expressing enhanced green fluorescent protein (eGFP), which can be obtained more easily than ESC-CMs and have comparable electrophysiological properties, Rubart et al showed an electrical integration after transplantation into healthy murine hearts by imaging Ca²⁺ transients with a 2-photon molecular excitation laser-scanning microscope. The quality of electrical integration, eg, the occurrence of conduction decelerations or blocks, and the electrophysiological maturation of the transplanted FCMs were not investigated.

The electrophysiological maturation of cardiomyocytes after transplantation was studied by Roell et al using cryoinjury as a model for myocardial infarction. Roell et al dissociated cryoinjured hearts at different time points after injection of eGFP-positive FCMs and performed current clamp recordings, which revealed a fetal action potential (AP) shape in early postoperation FCMs and an adult-like shape at a later date. Because hearts were dissociated before the measurements, no information about the electrical integration of the studied cardiomyocytes or microenvironmental conditions promoting the AP maturation was achieved.

Until now, although the capability to establish an electrical coupling with host cells has been demonstrated for transplanted cardiomyocytes, there are no data about the quality of electrical integration and about microenvironmental conditions required for the electrophysiological maturation of transplanted cells. Recently we developed a novel technique to prepare viable ventricular slices, which was applied in the present study to obtain new information about the electrophysiological maturation and integration of FCMs with regard to influences of the site of transplantation (cryoinjured tissue or adjacent myocardium). Understanding these mechanisms of cardiac cell therapy will provide a basis for future experiments involving specific modulations of cellular differentiation and function before transplantation, which will help to optimize the therapeutic efficiency as well as the safety of cardiac cell therapy.

Materials and Methods

Harvesting of FCMs

Ventricles were harvested from 12.5- to 15.5-day-old fetuses of transgenic HIM:OF1 mice expressing eGFP under control of the α-actinin promoter. Cells were dissociated and resuspended in DMEM supplemented with 20% FCS (Gibco/Invitrogen) at a concentration of 45 000 cells/μL. Subsequently, cells were either transplanted or cultivated for 6 days for control AP recordings.

Operation, Cell Injection, and Injection of Dyed Microspheres

Adult male HIM:OF1 wild-type mice were used as recipients. The surgical procedure and the induction of the cryoinjury were performed as described. Cells were injected subsequent to the induction of the cryoinjury in the cryoinjured area (450 000 cells/10 μL) and the adjacent myocardium (another 450 000 microspheres/10 μL). All experiments were approved by the local animal welfare committee.

Preparation of Ventricular Slices

Ventricular slices of operated animals were prepared 5 to 6 days after transplantation as described.

AP Recordings

Slices were examined on an inverted fluorescence microscope (Axiovert 200; Zeiss, Oberkochen, Germany), enabling the identification of eGFP-positive FCMs, which allowed an exact positioning of electrodes for recording and stimulation. A defined heating frequency was applied using a unipolar stimulation electrode, which was placed in healthy recipient tissue as indicated in the figure. Intracellular AP recordings were performed in host tissue or areas of transplanted FCMs as indicated using conventional glass microelectrodes.

Because electrical excitation originated from healthy host tissue, we determined the temporal interdependency of stimulation artifacts and APs recorded intracellularly in transplanted FCMs as an indicator of an electrical integration. FCMs were considered not to be electrically integrated if there was no temporal interdependency of stimulation artifacts and (spontaneous) APs or if there were no APs but a stable resting membrane potential. As a matter of course, a 1:1 synchronicity of stimulation artifacts and contractions of healthy host tissue observed in the microscope was a precondition for the valuation as conduction block or lack of electrical integration.

Dye Injection

To specifically label the FCMs in which AP recordings were performed, the gap junction impermeable fluorescent dye tetramethylrhodamine dextran (M, 10 000; Molecular Probes) was injected by iontophoresis via the recording electrode. For these experiments, electrodes were filled with 1% tetramethylrhodamine dextran dissolved in 0.2 mol/L KCl. Before the extraction of the electrode from the cell, a depolarizing direct current was applied (2 nA, 2 to 5 minutes). After the measurements, eGFP and tetramethylrhodamine fluorescence of FCMs were examined within the viable slices with a fluorescence microscope, which allowed optical sectioning by means of the grid projection technique (Axiovert 200M with ApoTome; Zeiss).

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Microscopy of Slices: Structural Integration and Viability of Transplanted FCMs

Microscopic examinations of viable slices of recipient ventricles showed the structural integration of transplanted cells, which could be clearly identified on the basis of their green fluorescence (Figure 1), in both healthy and cryoinjured myocardium. The engraftment of FCMs was verified by immunostainings (Figure 1 in the online data supplement) showing eGFP- and α-actinin–positive cells, which were much smaller in comparison with eGFP-negative host cardiomyocytes. No differences in FCM morphology were found between cells embedded within healthy and cryoinjured myocardium. Background fluorescence of host tissue, which was assessed in slices of sham-operated hearts, was negligibly small, and no areas of intensified green fluorescence were observed.

Cryoinjured regions within the slices could be distinguished from vital host tissue by a changed tissue structure (Figure 1 and supplemental Figure II). Whereas vital tissue...
possessed a striated texture representing the alignment of adult cardiomyocytes, cryoinjured tissue could be identified by a more homogenous nonstriated texture. The identification of cryoinjured tissue by its characteristic texture was confirmed by immunostaining of α-actinin, which was absent in cryoinjured regions (supplemental Figure II).

To evaluate the expression of gap junctions, which are the structural correlate of an electrical coupling, connexin 43 immunostainings were made. Connexin 43 expression was found between FCMs, between FCMs and host cardiomyocytes, and between host cardiomyocytes (Figure 2 and supplemental Figure III). It was also observed that nonmyocytes were interposed between FCMs and host cardiomyocytes at sites of connexin 43 expression.

Viability and functional integration of transplanted FCMs within the slices could be evaluated by microscopy because occasional conduction blocks or spontaneous beats enabled a distinction of active contractions and passive movements. Beating of transplanted FCMs could be observed in healthy as well as cryoinjured regions. In slices of sham-operated hearts, no beating was observed in cryoinjured areas (15 slices of 4 hearts).

AP Recordings: AP Morphology of Transplanted FCMs and Adult Cardiomyocytes
For the evaluation of electrophysiological properties of transplanted FCMs, it was crucial to reliably impale FCMs. Because of the small thickness of the slices (150 μm), the tip of the electrode was visible through the slices, which facilitated the exact positioning of the electrode. To verify the specific impalement of FCMs, injections of tetramethylrhodamine dextran were performed subsequent to AP recordings (n = 5). Optical sections of the areas in which measurements were made demonstrated a colocalized eGFP and rhodamine fluorescence of single FCMs (Figure 3).

Intracellular AP recordings demonstrated the existence of viable and electrophysiologically intact transplanted FCMs, either surrounded by cryoinjured tissue (Figure 4) or connected to viable host myocardium (Figures 5 and 6). AP shape was dominated by the absence of phase 1 repolarization and the existence of a plateau phase, which is typical for FCMs and was not found in host tissue. The ratio of AP duration at 50% repolarization (APD50) versus AP duration at 90% repolarization (APD90) (APD50/90 ratio; expressed as percentage), which was calculated to quantify the course of repolarization (see the expanded Materials and Methods section in the online data supplement) was approximately 50% in FCMs (Table). In contrast, APs of host cardiomyocytes showed an intense phase 1 repolarization (APD50/90 ratio of 10% to 20%), which is characteristic for adult murine APs.

Early afterdepolarizations, which might indicate an impaired electrophysiology and a potential proarrhythmic risk, were observed in transplanted FCMs in 4 of 53 recordings. Delayed afterdepolarizations were not observed.

To exclude the occurrence of fetal-like AP morphologies in host cardiomyocytes caused by cryoinjury and injection,
control recordings in slices of sham-operated hearts (injection of dyed microspheres) were performed at the border zone of the cryoinjured area and in regions of injected microspheres (52 recordings in 19 slices of 4 hearts). Within the cryoinjury, no APs could be recorded at all. Both at the border zone of the cryoinjured area and in regions of injected microspheres, nonphysiological AP morphologies (25 recordings in 13 slices of 3 hearts), but no fetal-like APs, were found. APs of injured adult cardiomyocytes were characterized by spontaneous diastolic depolarizations as well as a low maximal diastolic potential, amplitude, and maximal upstroke velocity ($V_{\text{max}}$; Table). APD$_{50}$ and APD$_{50/90}$ ratio of injured adult cardiomyocytes were significantly different from characteristic fetal parameters (supplemental Table I).

**AP Recordings: Electrical Integration of Transplanted FCMs**

When transplanted cells were surrounded by cryoinjured tissue, an electrical integration was never found; ie, there was no temporal interdependency of stimulation artifacts (stimulation electrode placed in healthy host tissue) and APs recorded in areas of transplanted cells (Figure 4). Therefore these APs must be considered spontaneous. Occasionally, there were no APs but stable resting membrane potentials. In all cases, a temporal interdependency of stimuli and contractions of healthy host myocardium was confirmed by microscopy.

The capability of transplanted FCMs to integrate electrically into host myocardium was approved by multiple measurements showing a temporal interdependency of stimulation artifacts and APs of transplanted cells (Figures 5 and 6). A structural connection to viable host tissue was a precondition for an electrical integration of transplanted cells. Eighty-two percent of FCMs that were embedded in viable host myocardium were electrically integrated. The temporal interdependency of stimulation artifacts and APs of FCMs remained when contractions were inhibited by 30 mmol/L 2,3-butanedione monoxime (data not shown), indicating that excitation of transplanted cells was not stretch dependent but mediated by electrical coupling. Correspondingly, gap junction uncoupling by heptanol (see below) completely blocked the excitation of FCMs.

By increasing the stimulation frequency up to 10 Hz, the maximal frequency leading to a stable 1:1 coupling and excitation of transplanted FCMs was determined and varied between 1 and 10 Hz (Figure 7A). Above the maximal frequency, either sporadic or regular (eg, 2:1, 3:2) failures of...

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**Figure 3.** Labeling of an impaled FCM after the recording. Tetramethylrhodamine dextran was iontophoretically injected subsequent to the recording via the recording electrode to verify the impalement of a FCM. A, Optical section (eGFP fluorescence only) through an area of transplanted FCMs. A cut view through 14 superimposed optical sections, which displays the cell size in 3D, is shown above and at the right hand side of the dashed line (A and B). The cut lines are indicated by red arrows. B, Overlay of eGFP (green) and rhodamine (red) fluorescence. The colocalization of eGFP and rhodamine fluorescence (in the optical section as well as in the cut views) demonstrated that a transplanted FCM had been impaled. C, Typical fetal AP recorded before the dye injection.

**Figure 4.** Nonintegrated FCMs. A, AP recording in an area of transplanted FCMs that were surrounded by cryoinjured tissue. (Recording sites are depicted in Figure 1.) There was no temporal interdependency between stimulation artifacts (vertical lines) and spontaneous APs; ie, cells were not electrically integrated. APs possessed a typical fetal shape with a pronounced plateau phase. B and C, Control recordings in adult host tissue at stimulation frequencies of 2 and 10 Hz. Each stimulus provoked 1 AP (1:1 excitation of cardiomyocytes); ie, cells at the recording site were electrically coupled to cells at the stimulation site. APs had the characteristic shape of murine adult APs with a fast phase 1 repolarization.
excitation of FCMs occurred (Figure 6). The failures of excitation appeared despite a complete repolarization of the membrane potential, and AP frequencies of up to 10 Hz could be evoked in these transplanted FCMs by intracellular stimulation via the recording electrode (n=8; supplemental Figure IV). Hence, an insufficient conduction, rather than refractoriness, caused the failures of excitation of FCMs. Therefore, the maximal frequency can be considered to be an indicator of the quality of electrical integration. In healthy host tissue, the maximal frequency was generally 10 Hz and a constant 1:1 beating persisted, whereas conduction blocks were recorded in regions of transplanted cells.

AP Recordings: Maturation of AP Properties of Transplanted FCMs
To evaluate the effect of an electrical integration on the maturation of AP properties of transplanted FCMs, $V_{\text{max}}$, APD$_{50}$, APD$_{90}$, maximal diastolic potential, and amplitude were determined for nonintegrated and well integrated FCMs (maximal stimulation frequency leading to a 1:1 excitation of transplanted FCMs, $>2$ Hz) (Figure 7B and the Table). As controls, APs of healthy host cardiomyocytes (within slices of operated hearts), injured adult cardiomyocytes (within slices of sham-operated hearts), and dissociated FCMs, which were cultivated for 6 days, were recorded. The stimulation frequency of host cardiomyocytes, injured adult cardiomyocytes, and well-integrated FCMs included in the analysis was 2 Hz; nonintegrated and cultivated FCMs had a comparable spontaneous frequency of $2.2\pm0.4$ Hz and $2.5\pm0.2$ Hz.

APs of healthy recipient cardiomyocytes possessed characteristic properties of murine adult cardiomyocytes, ie, a high maximal diastolic potential, amplitude, and $V_{\text{max}}$, as well as a short APD$_{50}$ and APD$_{90}$. In contrast, APs of cultivated FCMs had a significantly (all $P<0.001$) lower maximal diastolic potential, amplitude, and $V_{\text{max}}$, as well as a longer APD$_{50}$ and APD$_{90}$. Parameters of nonintegrated transplanted FCMs showed no statistical differences in comparison with cultivated FCMs (all $P>0.05$; see supplemental Table I for exact probability values). In contrast, well-integrated transplanted FCMs had a significantly higher maximal diastolic potential, amplitude, and $V_{\text{max}}$, as well as a shorter APD$_{50}$ and APD$_{90}$ (all $P<0.001$). However, maximal diastolic potential, $V_{\text{max}}$, APD$_{50}$, and APD$_{90}$ of well-integrated FCMs still differed significantly from values of adult host cardiomyocytes.

To test whether the changes in AP morphology of well-integrated FCMs were caused by an electrophysiological maturation of FCMs, or rather imposed by electrically coupled adult cardiomyocytes, well-integrated FCMs were experimentally uncoupled by 2 mmol/L heptanol (n=6; supplemental Figure V). Heptanol completely abolished the electrical integration of previously well-integrated...
FCMs. Uncoupled FCMs maintained a stable resting membrane potential and did not generate APs spontaneously. APs evoked by intracellular stimulation via the recording electrode did not have the immature properties of primarily nonintegrated FCMs, but maintained the short APD$_{50}$ and APD$_{90}$ typical for well-integrated FCMs. APD$_{50}$ and APD$_{90}$ even decreased to 84.5$\pm$7.2% and 81.2$\pm$2.3% ($P=0.16$ and 0.01), and the amplitude decreased to 85.7$\pm$4.4% ($P=0.03$). These findings support a maturation of electrophysiological properties of well-integrated but not of nonintegrated transplanted FCMs.

Although well-integrated FCMs had a significantly shorter APD$_{50}$ and APD$_{90}$ as well as a higher $V_{\text{max}}$ amplitude, and maximal diastolic potential compared with non-integrated or cultivated FCMs, a distinct plateau phase was detectable in all types of FCMs, which was in clear contrast to healthy or injured adult cardiomyocytes. The APD$_{50}$/APD$_{90}$ ratio, which was calculated to quantify the presence of a plateau phase, showed no significant differences among well-integrated, nonintegrated, and cultivated FCMs but was more than 2-fold higher compared with healthy or injured adult cardiomyocytes. Hence, the

### AP Parameters

<table>
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<th>Cell Type</th>
<th>Host Cardiomyocytes</th>
<th>Well-Integrated FCMs</th>
<th>Nonintegrated FCMs</th>
<th>Cultivated FCMs</th>
<th>Injured Adult Cardiomyocytes</th>
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<tbody>
<tr>
<td>$V_{\text{max}}$ (V/sec)</td>
<td>156.7$\pm$12.9</td>
<td>99.9$\pm$8.8</td>
<td>14.5$\pm$2.9</td>
<td>12.1$\pm$3.0</td>
<td>59.3$\pm$10.4</td>
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<tr>
<td>APD$_{50}$ (ms)</td>
<td>7.9$\pm$0.6</td>
<td>32.1$\pm$1.9</td>
<td>71.4$\pm$8.3</td>
<td>70.2$\pm$5.4</td>
<td>18.3$\pm$1.5</td>
</tr>
<tr>
<td>APD$_{90}$ (ms)</td>
<td>83.3$\pm$4.5</td>
<td>69.9$\pm$3.4</td>
<td>121.5$\pm$14.9</td>
<td>126.8$\pm$8.9</td>
<td>94.1$\pm$4.1</td>
</tr>
<tr>
<td>APD$<em>{50}$/APD$</em>{90}$ (%)</td>
<td>101$\pm$0.8</td>
<td>48.4$\pm$3.1</td>
<td>58.7$\pm$3.8</td>
<td>55.7$\pm$3.0</td>
<td>19.9$\pm$1.5</td>
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<tr>
<td>Maximal diastolic potential (mV)</td>
<td>$-74.6\pm1.2$</td>
<td>$-67.8\pm1.2$</td>
<td>$-55.7\pm2.2$</td>
<td>$-53.1\pm2.5$</td>
<td>$-58.0\pm1.6$</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>87.0$\pm$2.6</td>
<td>84.4$\pm$1.9</td>
<td>58.5$\pm$3.4</td>
<td>62.8$\pm$3.6</td>
<td>59.7$\pm$2.5</td>
</tr>
<tr>
<td>No. of recordings (slices/hearts)</td>
<td>26 (20/13)</td>
<td>33 (19/10)</td>
<td>20 (14/10)</td>
<td>15</td>
<td>25 (13/3)</td>
</tr>
</tbody>
</table>

The data are means$\pm$SEM. Corresponding $P$ values are tabulated in supplemental Table I.
APD_{50/90} ratio reliably enabled the discrimination of fetal and adult cardiomyocytes.

**Discussion**

In the present study, we investigated the electrophysiological maturation and integration of FCMs transplanted in vivo in viable or cryoinjured myocardium. For the first time, a novel slice technique was used throughout this study, which enabled us to directly assess the quality of electrical integration, eg, the occurrence of conduction blocks at high stimulation frequencies, as well as influences of the site of transplantation (cryoinjured tissue or adjacent myocardium) on electrophysiological properties of the transplanted cells. It could be demonstrated that transplanted FCMs integrated electrically and developed more mature AP properties only if the cells were embedded in viable host myocardium.

**Slice Technique**

Ventricular slices are a representative model of cardiac tissue with preserved in vivo structure, which possesses intact electrophysiological properties at the single-cell level as well as a homogenous excitation spread at the tissue level. Thus, electrical coupling and electrophysiological properties of transplanted cells within ventricular slices are likely to be representative of the in vivo situation at the time of preparation. However, the small thickness of the slices limits the evaluation of cellular interactions in the third dimension and their potential influences on electrical coupling as well as electrophysiological properties of individual cells.

It was crucial for the evaluation of integration and maturation to ensure that FCMs were reliably impaled. The reliability of the measurements was verified by control measurements in slices of sham-operated hearts, which did not reveal any fetal-like APs. Moreover, tetramethylrhodamine dextran injections through the recording electrode resulted in a colocalized eGFP and rhodamine fluorescence, confirming the reliable impalement of FCMs.

Electrical Integration of Transplanted FCMs

Until now, cell transplantation studies mostly focused on evaluations of global cardiac function and histology. Only few in vitro and in vivo studies dealt with the electrical integration of transplanted cardiomyocytes, which is a fundamental mechanism of cardiac cell replacement. These studies demonstrated the capability of different types of cardiomyocytes (ESC-CMs and FCMs) to build gap junctions and to establish electrical couplings with recipient cardiomyocytes. However, to date, there has been no investigation of the quality of electrical integration with regard to conduction insufficiencies and of the impact of microenvironmental conditions on electrophysiological properties of transplanted cardiomyocytes.

Corresponding to previous findings of Rubart et al., electrical couplings of adult cardiomyocytes and FCMs after transplantation were confirmed in this study by functional and immunohistological investigations. Whereas hearts between 8 and 37 days after transplantation were investigated in the former study with a maximal stimulation frequency of 4 Hz, we demonstrated that the electrical integration can be sufficient as soon as 5 to 6 days after transplantation, even when challenged by high stimulation frequencies of up to 10 Hz. However, we observed conduction blocks in a number of slice preparations, thus an arrhythmogenic potential of transplanted FCMs must be taken into consideration. These blocks were likely located at the host–donor interphase, because no conduction blocks within the host tissue were observed, neither at more proximal nor at more distal sites of the path of excitation spread. However, detailed electrical mapping...
Electrophysiological Maturation of Transplanted FCMs

Although FCMs located within the cryoinjured tissue showed no differences of AP properties as compared with cultured FCMs, well-integrated FCMs underwent a significant shortening of APD_{50} and APD_{90} and an increase of V_{max}, amplitude, and maximal diastolic potential, matching electrophysiological properties of more mature murine cardiomyocytes. The shorter APD_{50} and APD_{90} as well as a stable resting membrane potential persisted after uncoupling of previously well-integrated FCMs by heptanol, pointing to a maturation of electrophysiological properties, ie, an altered expression of ionic channels, rather than a faster repolarization and stabilization of the membrane potential imposed by electrically coupled adult cardiomyocytes. Local electrotonic interactions may have influenced APs of well-integrated FCMs after gap junction uncoupling in the present study. However, a shortening of APs of transplanted FCMs was also found by Roell et al, who performed current-clamp recordings in FCMs obtained by dissociation of whole recipient hearts 11 days after operation, ie, in the absence of electrotonic interactions. These findings support the electrophysiological maturation of FCMs demonstrated in the present study. Because the location of FCMs before dissociation could not be determined by Roell et al, no conclusions regarding site-specific effects of cryoinjured or viable host tissue on the maturation could be drawn. Considering our findings, the measurements of Roell et al were most likely made in FCMs that were embedded in viable host myocardium before dissociation.

The observed changes of the AP morphology of well-integrated FCMs correspond well to perinatal morphological alterations of APs of native rodent cardiomyocytes, which are predominantly mediated by an increase of transient outward K\(^+\) current. Further investigations, including gene and protein expression analyses of transplanted FCMs, are required to reveal the exact molecular alterations underlying the observed AP maturation of well-integrated transplanted FCMs and to draw a comparison with the native electrophysiological maturation.

It should be noted that AP properties of well-integrated FCMs were still significantly different from those of host cardiomyocytes. We hypothesize that further maturation might be observed when measurements are done at later times than those applied in the present study (5 to 6 days after transplantation). The fact that the electrophysiological maturation was found in FCMs embedded in viable host myocardium but not in FCMs surrounded by cryoinjured tissue argues against an exclusive regulation of maturation by soluble and diffusible cytokines. Instead, a direct structural contact to viable myocardium, ie, a niche provided by intact cardiac tissue, is presumably essential for the maturation of transplanted immature cardiomyocytes. Elements of this niche might include an electrical coupling via gap junctions, nonelectrical direct cell–cell interactions, specific components of the extracellular matrix, or mechanical exercise. Future studies involving the transplantation of genetically modified cardiomyocytes, eg, FCMs of connexin-deficient mice, will be needed to further elucidate the constituents of this maturation niche and to optimize the conditions for an effective and safe cell replacement. It is not possible for FCMs to be used for cell replacement in patients; instead, they could serve as a model for ESC-CMs. Although electrophysiological properties of FCMs and ESC-CMs are comparable, it cannot be excluded that ESC-CMs behave differently, in some way, after transplantation. Therefore, future studies of integration and maturation of transplanted cardiomyocytes should also include ESC-CMs.
In conclusion, we demonstrate, for the first time, that a high degree of electrophysiological maturation and integration of transplanted FCMs is already achievable 5 to 6 days after transplantation. Electrophysiological maturation and integration, which are elementary mechanisms of a successful cell replacement, strongly depend on an embedment of transplanted cells in viable host myocardium.

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Disclosures
None.

References
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Expanded materials and methods

Harvesting of FCMs

Transgenic HIM:OF1 mice, which express eGFP under control of the α-actin promoter\(^1\), were mated and pregnant females were sacrificed by cervical dislocation 12.5 to 15.5 days post coitum. Whole fetal hearts were harvested and eGFP expression was verified under a fluorescence microscope. After removing the atria, ventricles were cut into small pieces and dissociated as described before\(^2\). After dissociation, cells were resuspended in DMEM supplemented with 20% FCS (Gibco/Invitrogen) at a concentration of 45,000 cells/µl. Subsequently, cells were either transplanted or cultivated for 6 days in DMEM plus 20% FCS for control action potential (AP) recordings.

Operation, cell injection and injection of dyed microspheres

The surgical procedure was performed as described before\(^3\,4\). Male HIM:OF1 wild-type mice (> 5 weeks old) were sedated by a narcotic gas mixture containing 5% isoflurane (Baxter, Munich, Germany), ~50% O\(_2\) and ~50% N\(_2\)O. Sedated mice were intubated and anesthetized by 1.5% isoflurane. A thoracotomy was performed and the apex of the heart was laid open. A cryoinjury was induced by pushing a copper probe (3 mm diameter), which was cooled in liquid nitrogen, to the left ventricle (3 times ~ 20 s at the same site).

Directly after induction of the cryoinjury, 10 µl of the cell suspension (450,000 cells/10µl) were injected in the cryoinjured area and another 10 µl in the adjacent myocardium using a 10 µl Hamilton syringe connected to a steel needle by a polyethylene tube. Instead of the cell suspension, dyed microspheres (10 µm diameter, blue CML polystyrene latex, Molecular Probes/Invitrogen) dissolved in DMEM plus 20% FCS were injected in the cryoinjured area.
(450,000 microspheres/10 µl) and the adjacent myocardium (another 450,000 microspheres/
10 µl) of control mice. Except for the injection, control mice were treated exactly in the same
way as mice that received cell injections. Subsequently, the thorax was closed, the inhalation
anesthesia was stopped and the mice were extubated. A postoperative analgesia by tramadol
(15 mg/kg s.c. after the extubation plus 1 mg/ml in the drinking water for 4 days;
Gruenenthal, Aachen, Germany) and caprofen (5 mg/kg/d s.c.; Pfizer, Karlsruhe, Germany)
was administered.

Preparation of ventricular slices

Ventricular slices of operated animals were prepared at days 5 to 6 after transplantation as
described previously5. Mice were killed by cervical dislocation and hearts were removed
quickly. The aorta was perfused retrogradely via a syringe with oxygenated ice-cold Ca\(^{2+}\)-free
Tyrode’s solution (composition in mmol/L: NaCl 136, KCl 5.4, NaH\(_2\)PO\(_4\) 0.33, MgCl\(_2\) 1,
glucose 10, hepes 5, 2,3-butanedione monoxime 30; pH 7.4 adjusted with NaOH; all
chemicals were obtained from Sigma-Aldrich, Taufkirchen, Germany). Ventricles were
separated, embedded in 4% low-melt agarose (Roth, Karlsruhe, Germany) and glued on a
specimen holder. Short axis slices (thickness: 150 µm) were cut using a microtome (DTK–
2000, Dosaka, Kyoto, Japan) and stored in the standard ice-cold Tyrode’s solution, which was
supplemented with 0.9 mmol/L Ca\(_2\)Cl. After 30 min, slices were transferred into DMEM
without serum (Gibco/Invitrogen). Before measurements were carried out, slices were kept in
DMEM for at least 30 min.

AP recordings

For electrophysiological measurements, slices were transferred to a recordings chamber,
which was heated to 37°C, perfused with DMEM without serum (Gibco/Invitrogen) and
bubbled with 95% O\(_2\) and 5% CO\(_2\) (pH 7.4; concentrations of inorganic salts in mmol/L:
CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.3, NaHCO₃ 44, NaCl 110, NaH₂PO₄ 0.9). 2,3-butanedione monoxime (30 mmol/L) and heptanol (2 mmol/L; both Sigma-Aldrich, Taufkirchen, Germany) were added for special experiments as indicated.

Slices were examined on an inverted fluorescence microscope (Axiovert 200 with filter set 13; Zeiss, Oberkochen, Germany) enabling a clear identification of eGFP positive transplanted cells, which allowed an exact positioning of electrodes for recording and stimulation.

A defined beating frequency was applied using a unipolar stimulation electrode⁶, which was placed in healthy recipient tissue as indicated in the figures. The stimulation electrode consisted of a glass electrode (tip diameter ~50 µm) filled with DMEM, which was mounted on a micromanipulator and connected to a Grass SD9 stimulator via an Ag/AgCl wire. Another Ag/AgCl electrode was used as ground electrode. Slices were stimulated with a frequency of up to 10 Hz by pulses of 0.5 ms to 5 ms duration.

Intracellular AP recordings were performed in host tissue or areas of transplanted FCMs as indicated using glass microelectrodes (20-50 MΩ when filled with 3 mol/L KCl). Signals were processed by a SEC-10LX amplifier (npi electronic, Tamm, Germany) and acquired with the Pulse software (HEKA, Lambrecht/Pfalz, Germany). Maximal upstroke velocity ($V_{\text{max}}$), AP duration at 50% and 90% repolarization (APD₅₀ and APD₉₀), maximal diastolic potential and AP amplitude were analyzed with a customized version of the Mini Analysis program (Synaptosoft, Fort Lee, USA). The ratio of APD₅₀ divided by APD₉₀ (APD₅₀/₉₀ ratio; in %) was used to quantify the course of repolarization. A high APD₅₀/₉₀ ratio (~50-60%) indicated a pronounced plateau phase, a low APD₅₀/₉₀ ratio (~10-20%) was an indicator of a fast phase 1 repolarization.

Since the electrical excitation originated from the healthy host tissue, we determined the temporal interdependency of stimulation artifacts and APs recorded intracellularly in transplanted FCMs as indicator of an electrical integration. FCMs were considered not to be
electrically integrated, if there was no temporal interdependency of stimulation artifacts and (spontaneous) APs or if there were no APs but a stable resting membrane potential. As a matter of course, a 1:1 synchronicity of stimulation artifacts and contractions of healthy host tissue observed in the microscope was a precondition for the valuation as conduction block or lack of electrical integration.

**Immunostainings**

Slices were fixed for 20 minutes at –20°C in 99% methanol and embedded in paraffin. Sections were prepared with a thickness of 5 µm. The primary antibodies were anti-connexin 43 (clone CXN-6, Sigma-Aldrich, 1:200), anti-GFP (Santa Cruz, 1:25) and anti-α-actinin (clone EA53, Sigma-Aldrich, 1:400). Secondary detection was performed with anti-mouse IgM Alexa Fluor 647, anti-rabbit IgG Alexa Fluor 488 and anti-mouse IgG1 Alexa Fluor 555 (Molecular Probes/Invitrogen). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich). Image acquisition and processing were done with an Axiovert 200 M and Axiovision 4.5 (Zeiss).

**Statistics**

Data were tested for statistical significance by paired (heptanol experiments) or unpaired (comparison of AP parameters of different cell types) Student’s t-test or, if normality test failed, by Mann–Whitney rank sum test. All data are presented as mean ± S.E.M.
Supplemental figures

Supplemental fig. 1. Engraftment of transplanted FCMs. The engraftment of FCMs was verified by GFP immunostaining (green). Transplanted FCMs formed a cluster that was embedded in healthy host myocardium. Transplanted FCMs were cross-striated and possessed a much smaller cell size as compared to host cardiomyocytes. Red: α-actinin, green: GFP, blue: nuclei.

Supplemental fig. 2. Identification of cryoinjured tissue. A: Bright field image of the border zone of cryoinjured and healthy tissue of a viable slice. The cryoinjured region could be distinguished from vital host tissue by a changed tissue structure. While the vital tissue possessed a striated texture representing the alignment of adult cardiomyocytes, the cryoinjured tissue had a more homogenous non-striated texture. B: Corresponding fluorescence image. A GFP positive area of transplanted cells was located at the border zone of cryoinjured and healthy tissue. C: The identification of the cryoinjured tissue by its characteristic texture was confirmed by immunostaining of α-actinin, which was absent in the cryoinjured region. Red: α-actinin, green: GFP, blue: nuclei.

Supplemental fig. 3. Immunostaining of connexin 43 in an area of FCMs integrated in healthy host tissue. The gap juction protein connexin 43 was expressed in a punctate pattern between FCMs (arrow heads) and between FCMs and host cardiomyocytes (arrows). Between host cardiomyocytes, connexin 43 was arranged in intercalated discs. A: Superimposed channels: Connexin 43 (red), eGFP (green), nuclei (blue) and auto-fluorescence showing the morphology of FCMs and host cardiomyocytes (gray). B-D: Single channels: EGFP (B), auto-fluorescence (C) and connexin 43 (D).
Supplemental fig. 4. Intracellular stimulation of suboptimally coupled FCMs. To exclude that the absolute refractory period of electrically integrated FCMs caused the failures of excitation, which were observed during extracellular stimulation in the host tissue, FCMs that showed failures of excitation were stimulated intracellularly via the recording electrode (n=8). In all cases, the cells could be stimulated intracellularly with clearly higher frequencies up to 10 Hz, pointing to a suboptimal electrical coupling, i.e. conduction blocks at the host-donor interphase, underlying the failures of excitation. A: Stimulation via the extracellular electrode placed in healthy host tissue. The FCM was electrically integrated, but failures of excitation occurred at a frequency of 2 Hz. B: Intracellular stimulation via the recording electrode evoked APs up to a frequency of 10 Hz. C: Stimulation artifact of the intracellular stimulation.

Supplemental fig. 5. Uncoupling of well-integrated FCMs by heptanol (2 mmol/L).

A: Control recording in a well-integrated transplanted FCM. “#” marks the stimulation artifact (stimulation electrode placed in healthy host myocardium) preceding the AP. B + C: Recording in the same FCM after complete uncoupling by heptanol. Stimulation via the stimulation electrode placed in healthy host tissue (B) did not evoke APs any more. The resting membrane potential remained stable. Stimulation via the intracellular recording electrode (C; “*” marks the stimulation artifact) evoked APs that retained the typical AP morphology of well-integrated FCMs and did not develop the more prominent plateau phase characteristic for primarily non-integrated FCMs (fig. 4 of the printed manuscript). Thus, the shorter APD$_{50}$ of well-integrated FCMs as compared to primarily non-integrated FCMs is rather due to a maturation of electrophysiological properties than a direct effect of the electrical coupling to adult cardiomyocytes.
**Supplemental table 1.** P-values of the AP parameters tabulated in table 1 of the printed manuscript. Non-significant differences of AP parameters are marked by a gray shading. CMs: cardiomyocytes

<table>
<thead>
<tr>
<th>P-values</th>
<th>$V_{\text{max}}$ (V/s)</th>
<th>APD$_{50}$ (ms)</th>
<th>APD$_{90}$ (ms)</th>
<th>APD$<em>{50}$/APD$</em>{90}$ (%)</th>
<th>Maximal diastolic potential (mV)</th>
<th>Amplitude (mV)</th>
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<tr>
<td>Host CMs vs. well-integrated FCMs</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>Host CMs vs. injured adult CMs</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>Non-integrated vs. cultivated FCMs</td>
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<td>Non-integrated FCMs vs. injured adult CMs</td>
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<td>0.228</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.083</td>
<td>0.476</td>
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References


Supplemental fig. 1
Supplemental fig. 2

A

B

C

Healthy host myocardium

Cryoinjured tissue

500 μm

500 μm

100 μm
Supplemental fig. 4

A: Stimulation in host tissue, 2 Hz

B: Intracellular stimulation, 10 Hz

C: Stimulation artifact