Electrophysiological integration and action potential properties of transplanted cardiomyocytes derived from induced pluripotent stem cells

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Aims
Induced pluripotent stem cell-derived cardiomyocytes (iPSCM) are regarded as promising cell type for cardiac cell replacement therapy. We investigated long-term electrophysiological integration and maturation of transplanted iPSCM, which are essential for therapeutic benefit.

Methods and results
Murine iPSCM expressing enhanced green fluorescent protein and a puromycin resistance under control of the a-myosin heavy chain promoter were purified by antibiotic selection and injected into adult mouse hearts. After 6–12 days, 3–6 weeks, or 6–8 months, viable slices of recipient hearts were prepared. Slices were focally stimulated by a unipolar electrode placed in host tissue, and intracellular action potentials (APs) were recorded with glass microelectrodes in transplanted cells and neighbouring host tissue within the slices. Persistence and electrical integration of transplanted iPSCM into recipient hearts could be demonstrated at all time points. Quality of coupling improved, as indicated by a maximal stimulation frequency without conduction blocks of 5.77 ± 0.54 Hz at 6–12 days, 8.98 ± 0.38 Hz at 3–6 weeks and 10.82 ± 1.07 Hz at 6–8 months after transplantation. AP properties of iPSCM became more mature from 6–12 days to 6–8 months after transplantation, but still differed significantly from those of host APs.

Conclusion
Transplanted iPSCM can persist in the long term and integrate electrically into host tissue, supporting their potential for cell replacement therapy. Quality of electrical integration improves between 6–12 days and 6–8 months after transplantation, and there are signs of an electrophysiological maturation. However, even after 6–8 months, AP properties of transplanted iPSCM differ from those of recipient cardiomyocytes.

Keywords
Conduction • Electrophysiology • iPSCM • Transplantation

1. Introduction
Myocardial infarction and subsequent ischaemic heart failure are leading causes of mortality and morbidity. The almost irreversible loss of cardiomyocytes after myocardial infarction could not be overcome to date by pharmacological, interventional or surgical therapy. Therefore, exogenous cell replacement by transplantation of pluripotent stem cell-derived cardiomyocytes is considered a promising future therapeutic strategy.

To date, cardiac cell replacement therapy using pluripotent stem cell-derived cardiomyocytes has not been applied in clinical studies due to a number of potential complications, including immune rejection, tumorigenesis and arrhythmias, which need to be circumvented by thorough pre-clinical investigations. Moreover, there is an ongoing ethical debate especially on the use of embryonic stem cells.

Purification of differentiated cardiomyocytes by efficient lineage selection reduces the risk of tumour formation.1 The establishment of induced pluripotent stem (iPS) cell lines from differentiated somatic
2. iPS cell culture

2.1 iPS cell culture

The murine iPS cell line TiB7.4 was generated from murine embryonic fibroblasts (MEF) isolated from 129S4/SvJaeJ × C57Bl/6 mice and was kindly provided by Rudolf Jaenisch and Alexander Meissner.\(^{15}\) Cells were transfected with a plasmid vector containing the PAC-encoding puromycin aminopeptidase (PAC) and a reporter gene for puromycin resistance (gfp) under control of the α-muscle heavy chain promoter, as described previously for murine embryonic stem cell line α-PG44.\(^{1}\) iPS cells were grown on inactivated MEF in Dulbecco’s modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 15% foetal calf serum, 1 × non-essential amino acids, 2 mM l-glutamine, 50 μM β-mercaptoethanol, and 1000 IU/mL leukaemia inhibiting factor (Merck Millipore, Billerica, MA, USA).\(^{16,17}\) MEF were prepared from 129S4/SvJaeJ × C57Bl/6 mice and allowed to recover for another 30 min before AP recordings.

2.2 iPS cell differentiation and purification

For cardiac differentiation, 1 × 10⁶ iPS cells were suspended in 10 cm plates in 14 mL Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 20% foetal calf serum, 10 μM β-mercaptoethanol, 1 × non-essential amino acids and ascorbic acid (50 μg/mL), and placed on a horizontal shaker for 2 days to allow embryoid body formation. After 2 days, 27 500 embryoid bodies were transferred to a spinner flask filled with 200 mL of IMDM differentiation medium. At Day 9, puromycin (8 μg/mL) was added for cardiomyocyte purification, and medium with fresh puromycin was changed every second day. Purified iPS cells were harvested at Day 16, trypsinized and resuspended in IMDM for transplantation. The purity of cell population was analysed by cell cytometry (Attune® Cytometer, Applied Biosystems by Life Technologies).

2.3 iPS cell transplantation

Adult male wild-type 129S4/SvJaeJ × C57Bl/6 (Jackson Laboratory, Bar Harbour, ME, USA) mice were used as syngenic recipients. Surgery was performed as described.\(^{18}\) Briefly, animals were anaesthetized by inhalation of a mixture of nitrous oxide and oxygen (1:1) and isoflurane (1.5%; Baxter, Unterschleißheim, Germany). The adequacy of anaesthesia was assessed by testing tail and abdominal skin pinch reflexes with a blunt forceps. Two injections of 0.5 × 10⁶ iPS cells/10 μL were performed in the left ventricular wall. An intraoperative (tramadol, 15–20 mg/kg; sc; Grünenthal, Aachen, Germany) and postoperative (tramadol 0.2 mg/mL in the drinking water and caprofen 5 mg/kg/d sc for 4 days) analgesia was administered. All experiments were approved by the local animal welfare committee and conformed to the Directive 2010/63/EU of the European Parliament.

2.4 Preparation of vital ventricular slices

Recipients were sacrificed after 6–12 days, 3–6 weeks, and 6–8 months. Hearts were resected and tissue slices were prepared as described previously.\(^{19}\) Briefly, ventricles containing transplanted iPS cells were embedded in 4% low-melt agarose suspended in Tyrode’s solution (composition in mmol/L: NaCl 136, KCl 5.4, NaH₂PO₄ 0.33, MgCl₂ 1, α-glucose 10, HEPES 5, 2,3-butanediol monoxime 30; pH 7.4 adjusted with NaOH). Short-axis slices (thickness: 150 μm) were cut with a microtome (Leica VT1000S; Leica Microsystems, Wetzlar, Germany). Slices were stored in cold (4°C) Tyrode’s solution with 0.9 mol/L NaCl 2³⁻ for 30 min. Afterwards, slices were transferred to DMEM at 37°C aerated with 95% O₂ and 5% CO₂ and allowed to recover for another 30 min before AP recordings.

2.5 AP recordings and dye injection

Intracellular AP recordings were performed with sharp glass microelectrodes (15–40 MΩ) when filled with 3 mol/L KCl; World Precision Instrument, Sarasota, FL, USA) as described before.\(^{20}\) eGFP-positive iPS cells were identified by their green fluorescence, enabling a precise positioning of the recording electrode in graft or host tissue. A defined beating frequency was applied with a S9D square pulse stimulator (Grass Technologies, West Warwick, RI, USA) using a unipolar custom made stimulation electrode. Signals were amplified with an SEC-10LX amplifier (npi electronic, Tamm, Germany) and acquired with the Pulse software (HEKA, Lambrecht/Pfalz, Germany). Data were analysed offline with Mini Analysis (Synaptosoft, Fort Lee, USA).

Because electrical excitation originated from host tissue, we determined the temporal interdependency of stimulation artefacts and APs recorded intracellularly in transplanted cardiomyocytes as indicator of an electrical integration. iPS cells were considered not to be electrically integrated, if there was no temporal interdependency of stimulation artefacts and (spontaneous) APs or if there were no APs but a stable resting membrane potential. The quality of electrical integration could be assessed by the maximal stimulation frequency without conduction blocks, i.e. the maximal stimulation frequency leading to a 1:1 generation of APs after every stimulus. Only APs fulfilling the following specific conditions were included in the analysis of
the maximal stimulation frequency without conduction blocks to avoid falsely diminished values:

1. Host cardiomyocytes could be stimulated with a frequency of 10 Hz.
2. The structural integrity of the host tissue as well as the border zone of host and transplanted cells was confirmed by microscopy.
3. There was no fusion of stimulation artefacts and AP upstrokes, i.e. a direct stimulation of transplanted cells by the electric field of the stimulation electrode was excluded.

To further quantify the quality of electrical integration, we calculated the delay of electrical activation, i.e. the delay between stimulus and onset of the AP upstroke.

To label analysed cells, dextran-coupled tetramethylrhodamine (M, 10,000; Molecular Probes/Invitrogen, San Diego, CA, USA) was injected by iontophoresis via the recording electrode as described previously for transplanted foetal cardiomyocytes.13

2.6 Immunohistochemistry

Heart tissue was either cryo-conserved (anti-connexin 43 staining) or fixed for 90 min in 4% paraformaldehyde (PFA) and embedded in paraffin (other stainings). Sections were prepared with a thickness of 5 μm. Cryo-sections for connexin 43 staining were fixed with 4% PFA for 5 min. The primary antibodies were anti-GFP (rabbit IgG, A11122, 1:200; Molecular Probes/Life Technologies), anti-α-actinin (mouse IgG1, clone EA53, 1:400; Sigma-Aldrich, St Louis, MO, USA) anti-troponin I (mouse IgG2b, clone 284, 1:800; Millipore) and anti-connexin 43 (mouse IgM, C8093, 1:750, Sigma-Aldrich).

Secondary detection was performed with anti-rabbit IgG Alexa Fluor 488, anti-mouse IgM Alexa Fluor 647, anti-mouse IgG Alexa Fluor 555, and anti-mouse IgM Alexa Fluor 594 (Molecular Probes/Life Technologies). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich). Image acquisition and processing were done with an Axiovert 200 M microscope and Axiovision 4.5 (Zeiss, Oberkochen, Germany).

2.7 Statistics

All data are presented as mean ± SEM. Differences of AP parameters were tested for statistical significance by one-way ANOVA with post-test or, if normality test failed, by one-way ANOVA on ranks with post-test. All other data were tested for statistical significance by unpaired Student’s t-test or, if normality test failed, by Mann–Whitney rank sum test. A two-sided P-value <0.05 was considered statistically significant. SigmaStat (Systat, Erkrath, Germany) was used for all calculations.

3. Results

3.1 Microscopic and immunohistological evaluation of tissue slices

Before transplantation, the purified iPSCM population contained >95% eGFP-positive cardiomyocytes as assessed by flow cytometry. Transplanted cells could be easily identified within the slices by their green fluorescence (Figure 1A). Transplanted cells were arranged in clusters. Synchronous beating of graft and host tissue as well as conduction blocks were clearly visible under the microscope. Spontaneous contractions of transplanted iPSCM could also be observed. Since slices were to some extent transparent, the tip of the recording pipette could be localized by the inverted microscope used, enabling a specific positioning of the recording pipette in clusters of transplanted cells.13,14 To verify impairment of iPSCM, some analysed cells were labelled with the fluorescent dye tetramethylrhodamine via the recording electrode, resulting in red/green dual fluorescence (Supplementary material online, Figure S1).

Transplanted iPSCM were smaller than host cardiomyocytes and more polymorphic compared with the characteristic rod shape of adult host cells. Staining of cardiac troponin I showed cross-striation in transplanted iPSCM starting at Day 9 after transplantation with a lower degree of structural organization as compared with host cardiomyocytes (Figure 2A). At Day 6, we did not observe a clear sarcomere structure in transplanted iPSCM yet. Connexin 43 was arranged in intercalated disks between host cardiomyocytes and in a more punctual pattern between transplanted iPSCM. At the border between iPSCM and host cardiomyocytes, connexin 43 expression was scarce. Transplanted iPSCM and host cardiomyocytes were sometimes separated by non-cardiomyocytes (Figure 2B).

3.2 Electrical integration

Electrical coupling of iPSCM and host cardiomyocytes could already be demonstrated 6–12 days after transplantation (Figure 3), it was found in 33% of all measurements at Day 6 (n = 9) and 80% at Day 12 (n = 5) after transplantation. Later on, at 3–6 weeks (n = 8) and 6–8 months (n = 9) after transplantation, electric coupling was observed in 100% of all measurements.

The maximal stimulation frequency without conduction blocks, which was regarded as parameter of the quality of electrical integration, was 5.99 ± 0.51 Hz at 6–12 days, 8.98 ± 0.38 Hz at 3–6 weeks and 10.82 ± 1.07 Hz at 6–8 months after transplantation in coupled iPSCM (Figure 4A). Host tissue was able to follow a stimulation frequency of 11.84 ± 0.47 Hz (P < 0.001 vs. 6–12 days, P < 0.05 vs. 3–6 weeks, P = 0.760 vs. 6–8 months). Transplanted iPSCM, which had conduction blocks at frequencies <10 Hz when host tissue was focally stimulated, could be stimulated by field stimulation with a frequency of 10 Hz (Supplementary material online, Figure S2).

To quantify decelerations of excitation spread, we calculated the delay of electrical activation (Figure 4B). The delay of electrical activation amounted to 35.45 ± 5.66 ms at 6–12 days, 13.25 ± 2.58 ms at 3–6 weeks, and 12.53 ± 1.87 ms at 6–8 months after transplantation. In host cardiomyocytes, the delay of electrical activation was 10.21 ± 1.04 ms, i.e. Phase 0 depolarization of iPSCM at 6–12 days began ~25 ms later than it presumably would have begun in optimally coupled host cardiomyocytes. The delay of activation did not differ significantly between iPSCM at 3–6 weeks or 6–8 months and host cardiomyocytes (all P > 0.05).

3.3 AP properties

At all time points, AP morphology differed considerably between iPSCM and host cardiomyocytes (Figure 18 and Table 1). APs were very immature in transplanted iPSCM at 6–12 days after transplantation, characterized by a low maximal diastolic potential (MDP), low amplitude, low upstroke velocity (V_{up}) and high ratio of AP duration at 50% (APD50) and 90% (APD90) repolarization (APD50/90) as compared with host cardiomyocytes. In iPSCM cultured in vitro, which were analysed at Day 16 of differentiation (i.e. the day cells were harvested for transplantation), AP properties were also very immature (Table 1).

There were signs of an electrophysiological maturation of transplanted iPSCM from 6–12 days to 3–6 weeks and 6–8 months after transplantation (Figure 5 and Table 1). MDP, amplitude, and V_{up} increased from 6–12 days to 6–8 months after transplantation, APD50 and APD90/90 ratio decreased. While MDP and APD50 were not statistically different between host cardiomyocytes and iPSCM at 6–8 months after transplantation, a considerable difference of
amplitude, $V_{\text{max}}$, and APD50/90 ratio between host cardiomyocytes and iPSCM remained.

4. Discussion

In the present study, we characterized the electrical integration and AP properties of transplanted iPSCM for the first time. Using ventricular slices of recipient hearts, we were able to demonstrate that transplanted iPSCM survive in the long term and can couple electrically to host cardiomyocytes, which is a prerequisite for a successful cell replacement therapy. However, there were considerable differences between AP properties of graft and host, which may bear a proarrhythmic risk.

4.1 Cardiac cell therapy using iPS cells

Several reports on cardiac cell therapy using undifferentiated IPS cells\textsuperscript{6–9} and cardiac progenitor cells\textsuperscript{10,11} or cardiomyocytes\textsuperscript{12} derived from IPS cells have been published yet. In all these studies, a survival and structural integration of transplanted cells was described. It was shown that $\alpha$-actinin, a cardiac-specific protein, is expressed in the graft.\textsuperscript{8,10,12} Connexin 43 was found between transplanted iPSCM themselves and at the host–iPSCM interface.\textsuperscript{8,12} A functional benefit of IPS cell transplantation on the whole heart level was revealed by echocardiography, which was attributed to the de novo generation of cardiovascular tissue found in histology.\textsuperscript{8} Electrical coupling between graft and host was not assessed functionally.

4.2 Electrical integration

A high quality of electrical integration of transplanted iPSCM is essential for the safety and efficiency of cell therapy, since a block or deceleration of excitation spread may lead to malignant arrhythmias and hamper a synergistic contraction of graft and host. We were able to demonstrate that iPSCM have the capability to couple electrically to host tissue. The quality of coupling improved with time and became very good in coupled cells after 6–8 months, as indicated by a high-maximal stimulation frequency without conduction blocks and low delay of electrical activation.

On the other side, early after transplantation some cells were not electrically coupled at all, and conduction blocks and decelerations were frequent in electrically integrated iPSCM, as reflected by a mean maximal stimulation frequency without conduction blocks of $\approx 6$ Hz and a mean delay of excitation spread from host to graft tissue of $\approx 25$ ms. This may lower the safety and efficiency of cell therapy, at least transiently after transplantation.

iPSCM within the same cluster of transplanted cells usually had a comparable quality of integration. We did not find integrated and non-integrated iPSCM within the same cluster. In contrast, within one individual slice or several slices of one recipient heart, differences in the quality of coupling of transplanted iPSCM could be observed, i.e. clusters of well-integrated and non-integrated iPSCM could be found in an individual slice or heart.

Failure of excitation of transplanted iPSCM was rather caused by conduction blocks than by the refractory period of the cells, because cells were repolarized at the time of failing excitation. Moreover, transplanted...
cells could be stimulated via field stimulation with higher frequencies, if excitation via the surrounding host tissue failed.

Several studies on the electrical integration of transplanted cardiomyocytes have been published, using cardiomyocytes derived from human ES cells (ESCM)\textsuperscript{21,22} or foetal cardiomyocytes.\textsuperscript{13,14,18,23} The ability of transplanted cardiomyocytes to couple to host tissue was shown in all these studies. In line with the delay of electrical activation found in iPSCM early after transplantation in the present study, two-dimensional mapping data showed that the conduction velocity in areas of transplanted foetal cardiomyocytes and ESCM can be lower than in native tissue.\textsuperscript{21,22,24} On the other hand, the velocity of excitation spread in control hearts (transplantation of myoblasts or sham injections in injured hearts) was even lower, and the incidence of ventricular tachycardia was much higher in control hearts.\textsuperscript{21,24}

Human ESCM are able to follow stimulation frequencies of $\geq 5$ Hz after xenogenic transplantation in healthy recipients and $\sim 4$ Hz...
after transplantation in injured hearts. However, a comparison of maximal stimulation frequencies without conduction blocks between human ESCM and murine iPSCM is hampered by quite different electrophysiological properties of human and murine cardiomyocytes, especially the longer AP duration of the former. In murine foetal cardiomyocytes, 26% of transplanted cells at Day 6 and 53% at Day 12 had no conduction blocks at a stimulation frequency of 10 Hz, suggesting a faster progression of electrical integration as compared with iPSCM. This may indicate inherent functional differences between foetal cardiomyocytes and iPSCM. However, in contrast to iPSCM, foetal cardiomyocytes were not purified before transplantation, and the co-transplantation of non-cardiomyocytes may have contributed to the differences in the quality of coupling. In this case, co-transplantation of non-cardiomyocytes (e.g. fibroblasts, endothelial cells) could be a promising future strategy to improve the quality of coupling between iPSCM and host tissue.

**Figure 3** Electrical integration of transplanted iPSCM. (A) Fluorescence image of a heart slice depicting the position of stimulation and recording electrodes. (B and C) AP recordings in an electrically integrated iPSCM 9 days after transplantation (B) and in surrounding host myocardium (C). The iPSCM showed no conduction blocks at stimulation frequencies of 2 and 6.5 Hz, i.e. each stimulus triggered one AP. When the stimulation frequency was increased to 7 Hz, a 2:1 conduction block occurred. The surrounding host myocardium was able to follow a stimulation frequency of 10 Hz without conduction blocks. (D) AP recordings in an electrically integrated iPSCM 8 months after transplantation, showing no conduction blocks at 10 Hz. Please note the differences of AP morphologies recorded in iPSCM and host cells. See Figure 4 for corresponding statistics.
Apparently, Connexin 43 at the host-iPSCM interface was only expressed at very low levels, and graft and host cardiomyocytes were sometimes separated by a layer of non-cardiomyocytes. This likely contributed to conduction blocks observed in this study. The same histological observations have been made before by other groups.24,25

4.3 AP properties

APs of transplanted iPSCM differed considerably from APs of host cardiomyocytes at all time-points studied, although we found signs of electrophysiological maturation between 6–12 days and 6–8 months after transplantation. Inhomogeneities of AP duration can contribute to re-entry circles and cause severe arrhythmias.26 In one publication, an increase in ventricular tachyarrhythmias has been described after transplantation of ESCM.27 The authors did not investigate electrophysiological properties of cells after transplantation, but there were pronounced electrophysiological differences between host cardiomyocytes and ESCM before transplantation, which may have contributed to the observed arrhythmias. Thus, ESCM may have insufficiently matured like iPSCM in the present study. In contrast, two other studies demonstrated that ventricular arrhythmias can even be prevented by transplantation of ESCM as compared with controls (transplantation of myoblasts, ES cell-derived non-myocytes or sham injection).21,22

Unlike iPSCM, foetal cardiomyocytes show a fast maturation after transplantation and possess adult-like AP properties after 12 days, if they are electrically integrated.14 The maturation process of foetal cardiomyocytes is mainly characterized by a decrease in APD50 and an increase in APD90, leading to the typical shape of the repolarization phase of adult murine cardiomyocytes with a low APD50/90 ratio. In transplanted iPSCM, APD50 was already low early after transplantation and further decreased to values similar to adult cardiomyocytes after 6–8 months, but APD90 also decreased in the course of time, resulting in a high APD50/90 ratio even after 6–8 months.

According to their fast electrophysiological maturation, arrhythmias after transplantation of foetal cardiomyocytes have not been described, but could even be reduced. Like the differences in coupling of foetal cardiomyocytes and iPSCM to host tissue, the contrasting finding regarding the maturation of transplanted foetal cardiomyocytes and iPSCM may either be caused by an inherent difference between native cardiomyocytes and iPSCM, or by non-cardiomyocytes that have been transplanted together with foetal cardiomyocytes but not with purified iPSCM.

Figure 4: Quality of electrical integration of transplanted iPSCM. (A) The maximal stimulation frequency without conduction blocks was regarded as an indicator of the quality of electrical integration. Left: the mean maximal stimulation frequency was 5.99 ± 0.51 Hz 6–12 days after transplantation, 8.98 ± 0.38 Hz 3–6 weeks after transplantation, 10.82 ± 1.07 Hz 6–8 months after transplantation, and 11.97 ± 0.61 Hz in host cardiomyocytes. Right: histogram showing the percentage of cells that were able to follow a given frequency without blocks. The quality of electrical integration of transplanted iPSCM improved significantly from 6–12 days to 3–6 weeks and 6–8 months after transplantation. (B) The delay of excitation, i.e. the delay between stimulation artefact (SA) and AP upstroke (right, arrows), was assumed to be another indicator of the quality of electrical integration. In iPSCM, it was 35.45 ± 5.66 ms 6–12 days after transplantation, 13.25 ± 2.58 ms 3–6 weeks after transplantation and 12.53 ± 1.87 ms 6–8 months after transplantation. In adjacent host cardiomyocytes (left), it was 10.21 ± 1.04 ms, i.e. there was a mean delay of excitation between host and graft of ≈25 ms at 6–12 days after transplantation, but no significant delay at later time points. **p < 0.001; *p < 0.05.
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4.4 Limitations of the study

In the present study, iPSCM were injected into healthy syngenic adult hearts to study electrical integration and AP properties of transplanted iPSCM. This model was chosen, since an electrical integration and maturation of foetal cardiomyocytes has been described only for transplanted cells with structural contact to healthy host tissue, but not for transplanted cells embedded in cryo-injured tissue. Therefore, we did not induce myocardial injury in the present study. Since maturation of transplanted foetal cardiomyocytes was similar after injection in completely healthy hearts and healthy areas of cryo-injured hearts (unpublished data, Baumgartner S et al.), the results of the present study might be representative for transplanted iPSCM within healthy areas of injured hearts.

Viable ventricular slices enable detailed electrophysiological recordings in a preserved, in vivo like tissue structure. Slices of adult murine hearts possess physiological AP properties and show a fast spread of electrical excitation. Slices of recipient hearts have proved to be a reliable model to study electrophysiological properties of transplanted cells. Nevertheless, ventricular slices do not exactly match the in vivo situation, since slices are only 150 μm thick, kept in artificial medium and could be stunned or damaged by the preparation. Excitation spread may thus be hampered, and the quality of electrical integration may have been underestimated by our experiments.

AP properties of iPSCM could have been affected by the preparation, too, which may have led to an underestimation of electrophysiological maturation. We tried to minimize bias of preparation-related tissue damages by only including slices in our analyses that fulfilled specific quality criteria, i.e. host tissue followed a stimulation frequency of 10 Hz and the structural integrity of host myocardium and graft–host border zone was confirmed by microscopy.

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