Physiological Differences Between Transplanted and Host Tissue Cause Functional Decoupling after *in vitro* Transplantation of Human Embryonic Stem Cell-Derived Cardiomyocytes

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Key Words

Human embryonic stem cells • Xenogeneic stem cell transplantation • Engraftment • Cardiac myocyte • Arrhythmia • Electrophysiology • Action potentials • Beta-adrenergic agonists

Abstract

Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) might provide cells to repopulate injured myocardium. Electrical coupling of these cells to the host myocardium is a prerequisite for improved functionality. The aim of this study was to investigate electrical interaction of hESC-CMs with myocardial tissue and to identify factors challenging functional integration. Beating clusters containing hESC-CMs were cocultured *in vitro* with viable slices of late-stage embryonic murine ventricles. Field potentials recorded with micro-electrode arrays and video data were analyzed. The effects of heptanol, electrical pacing, beta-adrenergic, and muscarinic stimulation on coupling were studied. Beating clusters integrated morphologically and functionally resulting in a synchronized beating pattern after two to four days of

coculture. Heptanol-induced conduction block between transplanted cells and host tissue and immunoreactivity for connexin43 suggested electrical coupling *via* gap junctions. Beta-adrenergic or muscarinic stimulation induced uncoupling and arrhythmias probably due to genetically determined differences of hormonal modulation of spontaneous beating rates of transplanted cells and host tissue. HESC-CMs can integrate functionally and develop synchronized beating. Interventions unraveling the different electrophysiological properties of transplanted and host tissue induce functional disintegration. Successful cellular replacement has to improve coupling but should also aim to transplant cardiomyocytes with similar electrophysiological properties as the host tissue.

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Introduction

Transplantation of embryonic cardiomyocytes [1] or embryonic stem cell-derived cardiomyocytes (ESC-CMs) [2] has been shown to improve the function of the injured

heart (see [3] for review). Cardiomyocytes differentiated from pluripotent human cells, either cardiomyocytes derived from human embryonic stem cells (hESC-CMs) [4] or from pluripotent cells induced by reprogramming of differentiated human cells [5, 6] currently represent the most promising cell sources for cardiac regenerative medicine. HESC-CMs possess a typical cardiomyocyte structure *in vitro* [7, 8], show differentiation into multiple cardiac cell types [9], spontaneous activity [10, 11], hormonal modulation of chronotropy [10] and can develop and confer force to ischemically damaged myocardial tissue [12]. In addition to the morphological differentiation into cardiomyocytes, functional integration of transplanted cells and a synchronized beating behavior is likely to be a prerequisite for an effective improvement of cardiac pump function. Electrical interactions will also be critical for the arrhythmogenic potential of transplanted cells within the myocardium [13, 14].

Fundamental questions regarding time course of morphological and functional integration, arrhythmogenic potential, electrophysiological changes when hESC-CMs are coupled to the host tissue and the hormonal reactions of hESC-CMs after integration to physiological stimuli, e.g. hormones are far from being understood.

Furthermore, electrophysiological properties of cells for transplantation are likely to be different from adult human cardiomyocytes, either because they are still more immature, like progenitor cells, or are derived from cell lines with a different (e.g. atrial) phenotype. For these reasons, interaction of hESC-CMs and cell types with different electrophysiological properties needs to be investigated.

Technical limitations largely preclude repetitive measurements or pharmacological testing *in vivo* [15, 16]. In contrast, studies on a cellular level that have for example shown that hESC-CMs couple to neonatal rat cardiomyocytes *in vitro* [7] are of limited clinical relevance since they hardly reflect the complexity of cardiac structures *in vivo*.

In a recent paper, we demonstrated mechanical integration into cardiac tissue that had been subjected to simulated ischemia. Now, we modified the model and used vital tissue slices to investigate electrical integration [17]. Late-stage embryonic tissue slices provide the important aspect that their beating rate is very close to that observed in hESC-CMs [10] and human heart. In addition, murine electrophysiological properties at this developmental age resemble human cardiac tissue more closely than at any other age [18]. Thus, we could exclude simple overpacing by one structure and allow for a better obser-

vation of coupling under similar frequencies.

Materials and Methods

Culture of hESCs

The hESC line H1 (Wicell Research Institute, Madison, WI, USA, <http://www.wicell.org/>; passage numbers between 30 and 70) was imported according to German law and after approval by the Robert Koch Institute, Germany (No. AZ 1710-79-1-4-2). H1 cells were differentiated to spontaneously beating cardiac clusters as described previously [19]. Briefly, H1 colonies were cultivated on CF1 mouse feeder cell layers for approximately 7 days before they were transferred as clusters of approximately 500-800 cells onto layers of visceral endoderm-like cells (END-2) inactivated with mitomycin C (Serva GmbH, Heidelberg, Germany, <http://www.serva.de/>). HESCs were cultured in knockout Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA, <http://www.invitrogen.com/>) supplemented with 20% serum replacer (Invitrogen), 1 mmol/l L-glutamine, 1% non-essential amino acids, 0.09 mmol/l mercaptoethanol, 100 U/ml penicillin, 0.10 mg/ml streptomycin sulfate and 4 ng/ml basic fibroblasts growth factor (bFGF). HESCs and END-2 cells were cocultured for up to 2 weeks without serum or serum replacer and without bFGF, and scored for the presence of beating areas from 5 days onwards.

Preparation of ventricular slices

Generation of ventricular slices from late-stage embryonic murine hearts was performed as described previously [17, 20]. Briefly, late-stage embryonic mice (strain HimOF1 wild type) were killed at embryonic day 16.5. The hearts were excised rapidly and transferred to cold (4°C), oxygenated Ca²⁺-free Tyrode's solution containing 30 mmol/l 2,3-butanedione monoxime (BDM) to improve tissue preservation. Atria and heart valves were removed, ventricles were embedded in 4% low melting agarose (Carl Roth GmbH & Co. KG, Karlsruhe, Germany, <http://www.carl-roth.de/>) and sliced with a vibratome (DTK-1000, Dosaka EM Co., Ltd., Kyoto-shi, Japan, <http://www.kyoto.zaqq.ne.jp/dkaih504/>) in a plane parallel to the short axis resulting in ring-shaped slices of pure ventricular myocardium. The ventricular rings were stored in Ca²⁺-free, cold (4°C) Tyrode's solution (0.9 mmol/l Ca²⁺) for 30 min, transferred to Petri dishes containing cold Iscove's medium supplemented with 20% fetal calf serum without BDM, and warmed in an incubator (37°C; 5% CO₂) until coculturing with clusters containing beating hESC-CMs.

Coculture of ventricular slices with beating clusters containing hESC-CMs

Ventricular slices were plated on micro-electrode arrays (MEAs) on the day slicing was performed. Then, the excised hESC-derived clusters were placed partially beneath these slices providing direct contact to the ventricular slice (Fig. 1). Care was taken to assure that the hESC-derived cells covered electrodes in order to ease the detection of their relatively low-

Fig. 1. *In vitro* transplantation model. Schematic illustration of the hESC culture and coculture protocol. (a): Undifferentiated hESCs were cultivated on a CF1 feeder layer, (b): manually dissected and differentiated on END-2 feeder layers to beating clusters, (c): dissected again and (d): cocultured with viable myocardial tissue slices on MEAs. Late stage embryonic murine hearts (e) were cut orthogonal to the ventricular septum with a vibratome to get the tissue slices (f) that served as host tissue. Scale bars (b,c) 0.2 mm, (d) 0.4 mm, (e,f) 0.5 mm.

voltage FPs. Positioning at the outside margin of the coculture facilitated movement analyses. Videos and simultaneous FP recordings with MEAs were acquired on days 1-3 of coculturing.

Tissue processing, immunohistology and electron microscopy

For immunohistological staining, preparations were fixed for 120 min in 4% paraformaldehyde at room temperature, washed with PBS and incubated in 18% sucrose overnight prior to embedding in Tissue Tek OCT (Sakura Finetek Japan Co., Ltd., Tokyo, http://www.sakura-finetek.com/top_e.html). After cryoslicing (8 μ m), cryosections were placed on silanized slides. The following primary antibodies were used: anti-human nucleus (clone 235-1, 1:200, Chemicon International, Temecula, CA, USA, <http://www.chemicon.com/>), anti-connexin43 (clone CxN-6, 1:400, Sigma-Aldrich, St. Louis, USA, <http://www.sigmaaldrich.com/>), and anti-pan-cadherin (rabbit polyclonal, 1:400, Sigma-Aldrich). Anti mouse IgM Alexa Fluor 488, anti rabbit IgG Alexa Fluor 647 and anti mouse IgG1 Alexa Fluor 555 (Invitrogen GmbH, Karlsruhe, Germany, <http://probes.invitrogen.com/>) were used as secondary antibodies followed by nuclear staining with Hoechst 33342 (Sigma-Aldrich). Stained slices were imaged using an Axiovert 200, the image processing software was Axiovision Release 4.4 (both Carl Zeiss, Jena, Germany, <http://www.zeiss.com/>). For electron microscopy tissue was fixed in 4% paraformaldehyde for 120 min at room temperature. Preparations were then treated with 100 mmol/l Hepes/Pipes buffer containing 1% tannic acid for 30 min at room temperature and finally fixation was achieved with 0.5% OsO₄. Before embedding in Epon resin (Agar Scientific, Stansted, UK, <http://www.agarscientific.com/>) the tissue was dehydrated in a graded series of ethanol. Ultrathin sections (60 nm) were mounted on formvar-coated copper grids, stained with 0.2% uranyl acetate and lead citrate, and then examined with a Zeiss EM 902 A electron microscope.

Field potential (FP) recordings

Mapping of impulse propagation was performed as described recently [20]. Briefly, the timing of the maximal negative slope of the FP is used to estimate the local activation time. Temperature was regulated by a heating element and a tem-

perature sensor incorporated in the MEA amplifier (Multi Channel Systems, Reutlingen, Germany, <http://www.multichannel-systems.com/>) and adjusted to 37°C in all experiments.

Action potential (AP) recordings

For conventional micro-electrode recordings of transmembrane APs sharp glass electrodes filled with 3 mol/l KCl (tip resistance: 20-30 M Ω) were used. Signals were amplified by a SEC-10LX single electrode clamp amplifier (NPI Electronic, Tamm, Germany, <http://www.npielectronic.com/>) and acquired with the PULSE software (HEKA, Lambrecht/Pfalz, Germany, <http://www.heka.com/>). AP parameters were analyzed off-line with the Mini Analysis software (Synaptosoft, Decatur, GA, USA, <http://www.synaptosoft.com/>). All recordings were performed at 37°C in Tyrode's solution containing 1.8 mmol/l Ca²⁺.

Unless otherwise stated, chemicals were of high purity and purchased from Sigma-Aldrich.

Results

Morphological integration

After two to three days of coculture, excised beating clusters derived from hESCs attached firmly to the viable late-stage embryonic tissue slices. During this time, the initial hump created by the attached cluster of hESC-derived cells flattened and the border zone to the murine tissue smoothed partially because cells spread on the surface but also because they invaded into the tissue slice. Immunostaining with human-specific antibodies and an antibody against the endosarcomeric protein titin confirmed a high percentage of hESC-CMs as suggested by striations within the hESC-derived tissue (Fig. 2 a, b). In the transition zone, cells of murine and human origin were largely mixed (Fig. 2 a). Typically, the human tissue that infiltrated the murine slice was covered by a thin layer of titin-negative murine cells (Fig. 2 b). Connexin43 immunoreactivity was visible not only between cells of the same

Fig. 2. Morphological integration. (a-c): Transition zone between murine ventricular tissue and the beating hESC-derived cluster. (a): Anti-human nuclei antibody (red) identifies hESC-derived cells whereas nuclei stained solely with Hoechst dye (white) are of murine origin. Anti-titin (green) serves to identify sarcomeric structures. (b): hESC-CMs identified by anti-human mitochondria antibody (red) and a striated titin staining (green) covered by murine cells stained only by Hoechst dye (blue). (c): Connexin43 staining (white) is visible between human cells labeled with anti-human nuclei antibody (red) and murine cells whose nuclei are only counterstained by Hoechst dye (blue) and among these two cell types. (d): Ultrastructure of hESC-CMs with unspecific adhesion contacts to the murine tissue slice. (e,f): Heart-like cell-cell interactions with desmosomes (D), and fascia adherens (FA) within beating clusters containing hESC-CMs that still exhibit an immature phenotype with poorly organized contractile elements (black triangles indicating the cell-cell contact site). Scale bars (a,b) 0.05 mm, (c) 0.025 mm, (d-f) 1000 nm.

species but also between human and murine tissue (Fig. 2 c).

Ultrastructurally, we observed extracellular matrix and solely non-specific adhesion contacts between the murine and hESC-derived cells, i.e. cardiomyocytes (Fig. 2 d) or other cell types, including fibroblast-like cells. Differently, typical close cardiac cell-cell connections with intercalated disks, desmosomes and fasciae adherentes existed within the murine ventricular tissue and between the hESC-CMs (Fig. 2 e, f).

Functional integration

Spontaneous activity is typical of embryonic cardiac tissue. Synchronous beating of clusters containing hESC-CMs and the murine tissue slice, indicating functional integration, occurred in about 33% of cocultures. The success rate was primarily dependent on whether a sufficient mechanical contact between the human and murine tissue could be achieved and maintained during routine culture work. In cocultures where synchronicity could be analyzed on four consecutive days ($n=65$), and beating of both components could be observed ($n=21$, 43, 50 and 47 for days 1-4 of coculture) synchronous beating was never observed during the first day, in 7/43 (16%) cocultures on day 2, increased to 20/50 (40%) of cocultures on day 3 and 22/47 (47%) on day 4. Frequently, conduction blocks occurred, either sustained or intermittently (Fig. 3 a). Occasionally, we observed considerable

but usually stable conduction delays of up to 150 ms, in rare cases even of up to 400 ms between the murine and human tissues.

An analysis of the beating rate of cocultures where the cluster containing hESC-CMs and the tissue slice beat asynchronously showed significantly higher frequencies for the hESC-CMs as compared to the tissue slice one and two days after onset of coculture. Thereafter, though the mean frequencies of hESC-CM containing clusters still remained higher than the frequencies of the tissue slice, this difference became non-significant. Beating rates of synchronously beating cocultures were higher than the beating rate of asynchronously beating tissue slices and lower than those of asynchronously beating hESC-CM containing clusters (Fig. 3 b).

Impulse propagation was determined by the local activation time (time of maximal downstroke velocity, $dV/dt_{\max}$) as described previously [20]. To describe the spread of excitation, eight sector areas of equal size were defined for the elliptical coculture preparations (Fig. 3 c). In 49 maps generated from 42 preparations with a synchronous beating behavior, excitation of the tissue slice started most frequently (39%) in the two sectors close to the hESC-derived beating cluster and least frequently (16%) in the two sectors opposite to the hESC-derived beating cluster. The excitation wave front typically ended preferably in the four segments opposite to the hESC-derived cluster (31%) and least frequently in the two sec-

Fig. 3. Functional integration.

(a): Synchronicity of cocultures that showed beating of the hESC-derived and the murine slice. (b): Adaptation of the beating rate of murine tissue and hESC-derived beating clusters in coculture preparations. (c): Sections of the coculture preparation where the excitation of the murine slice started (top left) and ended (down right). (d): Position of stimulation electrode (S) and the two recording sites (I, II) for intracellular recording. (e): APs of the murine tissue slice and (f) corresponding APs of the hESC-derived tissue demonstrate a delayed onset of the upstroke in the transplanted area as compared to an almost instantaneous action potential upstroke of the tissue slice and a morphology that clearly identifies the different origin of the cells. Scale bar (d) 0.2 mm.

tors close to the hESC-derived beating cluster (18%) indicating that in the majority but not in all cocultures hESC-CMs paced the tissue slice and not *vice versa* (Fig. 3 c).

Electrophysiological phenotype after *in vitro* transplantation

AP recordings provided further evidence for a functional coupling between the tissue slice and the hESC-derived beating cluster. Electrical stimulation was achieved by micro-electrodes positioned within the center of the murine-derived slice. APs were recorded either at a site within the murine-derived slice or at the hESC-derived cluster (Fig. 3 d). These measurements consistently demonstrated the short AP duration of murine tissue slices and the broad AP of the hESC-CMs (n=7). A typical experiment is shown in Fig. 3 e: Although in this experiment, after four days of coculture, the distance of the two recording sites to the stimulation electrode was similar (range: 250 to 400 μ m), the onset of the APs recorded from the murine site (n=10 APs) was shortly after the stimulus (3.5 $\pm$ 0.1 ms) whereas it was significantly delayed (58.2 $\pm$ 0.2 ms, p < 0.05) for the APs recorded from the hESC-derived cluster. The APs recorded from the center of the murine slice were characterized by a fast maximal phase 0 upstroke velocity (dV/dt_{max} : 137.7 $\pm$ 0.1 V/s), an amplitude of 97.2 $\pm$ 0.1 mV and a resting potential (RP) of -61.3 $\pm$ 0.1 mV, and a short AP duration at 50% repolarization (APD 50; 37.7 $\pm$ 0.1 ms), i.e. parameters typical for embryonic murine tissue (Fig.

3 e). In contrast, APs recorded from hESC-derived tissue (n=10 APs) had a slower dV/dt_{max} (6.8 $\pm$ 0.6 V/s, p < 0.05), a smaller amplitude (71.6 $\pm$ 0.3 mV, p < 0.05), a higher RP (-52.6 $\pm$ 0.2 mV, p < 0.05), and a significantly longer APD 50 (100.7 $\pm$ 0.5 ms, p < 0.05), that is typical for hESC-CMs (Fig. 3 f).

During MEA recordings, FPs of murine tissue slices typically had a 25 to 30-fold higher amplitude (1275 $\pm$ 180 μ V, n=50, p < 0.05) and a higher maximal down stroke velocity as compared to those of hESC-derived clusters that were of low amplitude (46 $\pm$ 14 μ V, n=10).

Gap junction uncoupling

To analyze the mechanisms underlying synchrony, we administered the gap junction inhibitor 1-heptanol to synchronously beating coculture preparations (n=3) (Fig. 4 a, b). 1-Heptanol induced a complete conduction block between the human and the murine component at concentrations of 0.1-1 mmol/l and a reduced contractility as suggested by a reduction of the amplitude of the video-derived signal of both components (Fig. 4 c). In preparations where 1-heptanol induced a stop of slice beating while beating of the hESC-derived cluster continued (n=2), mapping of the local activation time indicated a spread of excitation originating close to the site of the hESC-derived beating cluster, a slowing of impulse propagation within the murine tissue with increasing concentrations of 1-heptanol and indicated that the excitation originated close to the site of the (Fig. 4 d).

Fig. 4. Functional integration: Uncoupling induced by the gap junction blocker heptanol. (a): The position of the hESC-derived cluster (green) at the outer surface of the coculture preparation allows differentiating its beating pattern from the contractions of the slice (red), (b) diagram showing the beating frequency and original traces from video analyses (c) show synchronous beating of the hESC-derived cluster (black) and the murine ventricular tissue slice. With increasing concentrations of 1-heptanol, an increasing degree of conduction block between the two structures is observed that can be washed out completely. (d): Activation sequence maps illustrate that excitation starts from an area close to the hESC-derived cluster and is slower with increasing concentrations of 1-heptanol. Scale bars (a) 0.1 mm, (d) 0.2 mm.

Sensitivity of coupling to changes of frequency

Since frequency-adaptation is an important physiological property of the heart, we analyzed how coupling was challenged by frequency changes ($n=4$) (Fig. 5). Whereas electrical stimulation of the center of a murine tissue slice (Fig. 5 a) always resulted in synchronous APs of the murine tissue up to a stimulation frequency of 10 Hz (i.e. the highest frequency tested), the hESC-derived cluster answered stimuli only below 1-6 Hz (Fig. 5 b-d). In addition, we analyzed whether frequency-dependent changes of the APD were influenced by coculturing. While APD 50 increased in the murine-derived tissue with increasing rates it was shortened in APs recorded from the hESC-derived cluster (Fig. 5 c, d) in 3/4 experiments and remained unchanged in a fourth preparation.

Hormonal modulation

Autonomous regulation is known to exhibit age- and species-typical differences [21]. We studied the influence of beta-adrenergic stimulation with (-)-isoproterenol (Iso)

and muscarinic stimulation with carbachol (Cch) in synchronously beating coculture preparations. The beating rate of the murine-derived slice of coculture preparations increased from 1.0 ± 0.3 Hz to a maximum of 2.9 ± 0.7 Hz 228 ± 43 s after administration of Iso ($n=6$) and induced a loss of synchrony in three coculture preparations. In control slices, Iso induced a maximal increase of the baseline rate from 2.3 ± 0.5 Hz to 3.8 ± 0.4 Hz after 85 ± 25 s ($n=4$).

Cch (0.05 mmol/l, 0.1 mmol/l in one coculture preparation) administered in addition to Iso to control slices without hESC-derived cells decreased the beating rate for 10-s intervals instantaneously to 0.1 ± 0.1 Hz (maximum inter-beat interval: median: 18 s, range: 6-813 s; $n=4$). The beating frequency of the murine part of coculture preparations decreased to 0.3 ± 0.2 Hz (maximum inter-beat interval: median 17 s, range: 2-4880 s; $n=6$), whereas the beating rate of the hESC-CMs in coculture preparations ($n=6$) only dropped to 0.6 ± 0.2 Hz with a median duration without contraction of merely

Fig. 5. Frequency-induced uncoupling. Electrically stimulated coculture preparation. (a): Localization of the stimulation site (dark gray dot) and the recording sites (light gray dots). (b): AP frequency of the murine (open triangles) and human tissue (filled dots) as a function of the stimulation rate. AP recordings (c) within the hESC-CM, (d) in a murine cardiomyocyte at different stimulation rates illustrate the markedly different AP shape, the delayed activation of the hESC-CM during stimulation of the murine slice and the higher stimulation rate accepted by the murine tissue. Whereas APD 50 increases with increasing frequency in mice, it decreases in the human tissue. Scale bar (a) 0.4 mm.

1.9 s (range: 0.7-5.5 s). The Cch effect recovered within 10 min in control slices as well as in both components of the coculture preparations. However, the spontaneous beating rate after 10 min remained below their baseline values in all control slices ($n=4$) and increased to values above their baseline beating rate in all hESC-derived clusters of the coculture preparations ($n=6$), whereas the murine slice of the cocultures recovered to values above ($n=4$) or remained below their baseline values ($n=2$).

A typical behavior of the modulatory effects of hormones is shown on a synchronously beating culture preparation in Fig. 6: Administration of Iso ($0.1 \mu\text{mol/l}$) resulted in an increase of the spontaneous beating rate of the murine part of the coculture as well as of the hESC-derived tissue. However, beating behavior became irregular, mainly due to a more pronounced increase of the spontaneous beating rate of the murine tissue as compared to the hESC-derived cluster. Cch ($50 \mu\text{mol/l}$) stopped spontaneous activity of the tissue slice completely for more than 15 s, but the hESC-derived section continued contracting, albeit at a transiently lower rate (Fig. 6 b, c left). During control conditions, the FP (Fig. 6 d left) of the hESC-derived tissue preceded the electrical activity of the slice. The corresponding activation sequence map (Fig. 6 e left) indicates that the slice activity originated close to the hESC-derived tissue, suggesting synchronous activity to be triggered by the human cells. Iso resulted in an

increased beating rate, primarily due to a high number of 'triple beats', i.e. a tissue slice FP, immediately followed by a FP of the hESC-derived tissue and a second FP of the slice (Fig. 6 d middle). These 'triple beats' are a reentry phenomenon since the slice FP preceding the FP of the hESC-derived tissue indicated an origin within the slice whereas the slice FP following the hESC-derived tissue originates close to the hESC-derived tissue (Fig. 6 e middle). After treatment with Iso and Cch FPs generated by the hESC-derived tissue and the murine tissue slice lacked temporal association (Fig. 6 d right) and the corresponding activation sequence (Fig. 6 d right) during these asynchronous contractions suggested a spontaneous activity of the slice distant to the area of the hESC-derived tissue. Thus, these data demonstrate that different reactions of both tissues to hormones can induce functional disintegration and could even unmask morphologically almost indistinguishable and functionally well-integrated areas of viable hESC-CMs.

Discussion

The two main results of the present study are that, even if functional integration of hESC-CMs into myocardial tissue can be achieved during controlled conditions, safety of conduction is limited and the electrophysiological

Fig. 6. Hormonal uncoupling. (a, b): Treatment of a synchronously beating coculture with Iso (100 nmol/l) resulted in a fast but irregular and asynchronous beating pattern. Additional Cch (0.05 mmol/l) transiently stopped the spontaneous contractions of the slice whereas the beating frequency of the hESC-CM-containing cluster slowed but recovered rapidly resulting in a coculture preparation with regular spontaneous contractions of the murine tissue and the hESC-CM-containing tissue at different frequencies. Scale bars (a,e): 0.2 mm.

phenotype of the transplanted hESC-CMs remains different.

Similarly to previous *in vitro* studies [7, 22], we could demonstrate staining for the gap junction protein connexin43 and functional relevance of coupling *via* gap junctions by heptanol-induced uncoupling of hESC-derived cells from the murine tissue slice. In contrast to aforementioned studies, we observed uncoupling of murine and human tissue by heptanol at concentrations where coupling between cells of the same species was still maintained. In addition, we frequently observed conduction blocks and temporal evolution of coupling took several days. This is in contrast to older studies that showed synchronization of embryonic chick heart cell aggregates [23] or cocultures of human ESC-CMs and dissociated

neonatal rat ventricular cardiomyocytes within hours [7, 22]. Since our results were confirmed if murine instead of human ESC-CMs were used for coculture [24], we can exclude species differences as an explanation. We assume that difficulties to couple might result from interposed non-cardiac, fibroblast-like cells derived from hESCs that we could identify in our previous studies [12] or from fibroblasts or endothelial cells derived from the myocardial tissue slice. These non-cardiomyocytes result in a structure that resembles a structure that has been described for the cardiac conduction system [25, 26]. Experimental studies have shown that these non-cardiomyocytes can modify beating rate and impulse propagation, and can cause electrical delay and block [27, 28]. Therefore, future studies to define the role of non-cardiomyocytes for cellular cardiomyoplasty would be highly relevant.

We observed uncoupling after administration of (-)-Iso (Fig. 6), whereas during recent hybrid culture studies with neonatal rat cardiomyocytes [7, 22] (+/-)-Iso had a positive chronotropic but no desynchronizing effect. Desynchronization by beta-adrenergic agonists may be explained by the known developmental differences of beta-adrenergic agonists for the regulation of chronotropy and its modulation of I_f channels [29, 30]. Partial decoupling resulted in desynchronization and severe irregularities of the beating behavior including rhythm disturbances resembling reentry (Fig. 6) which is a known pathophysiological substrate for cardiac arrhythmias [31]. The application of the muscarinic agonist Cch after prestimulation with Iso also had clearly different effects on the murine as compared to the human tissue, which could even unmask morphologically and functionally well-integrated hESC-CMs. Whereas in the transplanted early hESC-CMs Cch effects were mild and transient, negative chronotropic effects on the late-stage embryonic murine slice were pronounced and recovery was delayed or even absent. This is in line with previous studies in murine ESC-CMs showing that during early embryogenesis negative chronotropic effects of Cch are transient because they are predominantly mediated by the easily diffusible nitric oxide *via* the nitric oxide-cGMP-phosphodiesterase signal transduction cascade whereas muscarinic effects at later stages are largely due to a G_i -protein-mediated sustained down-regulation of adenylyl cyclase activity [32]. Therefore, a stimulation with beta-adrenergic or muscarinic agonists can result in a functional disintegration and constitute an arrhythmogenic substrate [31].

Intracellular recordings provide further evidence that

physiological properties of host and transplanted tissue remained different even after four days of coculture and even if functional coupling was achieved. Whereas APs recorded in hESC-CMs were characterized by long APDs with slow upstroke velocities, APs recorded from the murine slice were significantly shorter. Furthermore, the observed shortening of the APD with increased frequency is a well-known property of human [33] as opposed to murine tissue [34] and probably reflects differences in the expression of potassium channels [35].

In conclusion, in addition to the juxtaposition of phenotypically different cardiomyocytes which is known to be arrhythmogenic [31], we observed other arrhythmogenic substrates like reentry, conduction delay, and spontaneous activity.

Future studies will have to elucidate the mechanisms underlying functional integration. Reduction of the arrhythmogenic potential could be achieved by an improved coupling. Tissue slices generated from human cardiac tissue and genetically modified mice can be used to define the role of the extracellular matrix, lineage selection will help to delineate the specific role of different cell types, especially non-cardiomyocytes, and long-term experiments will have to show whether an improved functional integration can be achieved with time.

Our data support the concept that the electrophysiological phenotype of cells for regenerative purposes should be as close to the host's phenotype as possible. Cardiomyocytes isolated from different developmental stages of the human heart are known to differ substantially with respect to their electrophysiological behavior. I_{to1} and I_{K1} which are absent in hESCs were identified as markers of phenotypical cardiac differentiation. Early hESC-CMs have quite homogeneous APs and present a low density of I_{to1} and I_{K1} , a situation comparable to an atrial or a pacemaker phenotype [29]. hESC-CM reach more mature phenotypes over several months of cultivation but up to now a typical adult phenotype has not yet been attained, even several weeks after *in vivo*

transplantation [7, 8, 29, 36].

For this reason, future research efforts will also have to aim at generating hESC-CMs that have already acquired the electrophysiological phenotype of the host tissue *in vitro* or will have to show that transplanted cells differentiate into this phenotype *in vivo*. At the moment, it seems to be prudent to perform thorough investigations on functional integration and cardiac differentiation before testing hESC-CMs clinically.

Abbreviations

AP (action potential); APD 50/90 (action potential duration at 50/90% repolarization); Cch (carbachol); END-2 (visceral endoderm-like cells); ESC (embryonic stem cell); FP (field potential); hESC (human embryonic stem cell); hESC-CM (human embryonic stem cell-derived cardiac myocyte); Iso ((-)-isoproterenol); MEA (micro-electrode array); RP (resting potential).

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