Cardiac Myocytes Derived from Murine Reprogrammed Fibroblasts: Intact Hormonal Regulation, Cardiac Ion Channel Expression and Development of Contractility

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Key Words

Reprogramming • Pluripotency • Cardiomyocytes • Stem Cells • iPS

Abstract

Aims: Induced pluripotent stem (iPS) cells have a developmental potential similar to that of blastocyst-derived embryonic stem (ES) cells and may serve as an autologous source of cells for tissue repair, *in vitro* disease modelling and toxicity assays. Here we aimed at generating iPS cell-derived cardiomyocytes (CMs) and comparing their molecular and functional characteristics with CMs derived from native murine ES cells. **Methods and Results:** Beating cardiomyocytes were generated using a mass culture system from murine N10 and O9 iPS cells as well as R1 and D3 ES cells. Transcripts of the mesoderm specification factor *T-brachyury* and non-atrial cardiac specific genes were expressed in differentiating iPS EBs. Using immunocytochemistry to determine the expression and intracellular organisation of cardiac specific structural proteins we demonstrate strong similarity between iPS-CMs and ES-CMs. In line with a previ-

ous study electrophysiological analyses showed that hormonal response to β -adrenergic and muscarinic receptor stimulation was intact. Action potential (AP) recordings suggested that most iPS-CMs measured up to day 23 of differentiation are of ventricular-like type. Application of lidocaine, Cs⁺, SEA0400 and verapamil+nifedipine to plated iPS-EBs during multi-electrode array (MEA) measurements of extracellular field potentials and intracellular sharp electrode recordings of APs revealed the presence of I_{Na} , I_{F} , I_{NCX} and I_{CaL} , respectively, and suggested their involvement in cardiac pacemaking, with I_{CaL} being of major importance. Furthermore, iPS-CMs developed and conferred force to avitalized ventricular tissue that was responsive to β -adrenergic stimulation. **Conclusions:** Our data demonstrate that the cardiogenic potential of iPS cells is comparable to that of ES cells and that iPS-CMs possess all fundamental functional elements of a typical cardiac cell, including spontaneous beating, hormonal regulation, cardiac ion channel expression and contractility. Therefore, iPS-CMs can be regarded as a potentially valuable source of cells for *in vitro* studies and cellular cardiomyoplasty.

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Introduction

A major obstacle to successful clinical use of embryonic stem (ES) cell derivatives for tissue repair is the immunological rejection of mismatched cellular grafts. One solution to this problem would be to generate the patient's own "embryonic" stem cells by epigenetic reprogramming of his somatic cells to a pluripotent ES-cell-like state, from which other cell types required for therapeutic purposes could be generated. Fusion of different murine adult cells as well as human fibroblasts with ES cells has been shown to promote the epigenetic reprogramming of the adult genome to an embryonic phenotype [1, 2]. As a major disadvantage, these tetraploid fusion hybrids contain the DNA of both fusion partners and may lose chromosomes during prolonged culture. An alternative way of obtaining ES cell-like cells from somatic cells has been developed recently and involves the conversion of somatic cells into induced pluripotent stem (iPS) cells by overexpression of few pluripotency factors in somatic cells which endows them with the properties of ES cells [3, 4]. These iPS cells are able to form viable adult chimaeras, are germline-competent and their gene expression profile, DNA methylation status and chromatin configuration are similar to those of ES cells [4].

Reprogramming of human somatic cells using this approach has been reported by several groups [3, 5, 6]. Yamanaka's and Daley's group succeeded in converting human fibroblasts into an ES cell-like state by using the same four factors used in the murine model, while Thomson's group obtained similar results by using Oct4, Sox2, Nanog and Lin28 [7]. These human iPS cells also had all the essential properties typical of human ES cells, and differentiated into derivatives of all three primary germ layers, including beating cardiomyocytes (CMs). Second generation iPS were derived without integration of transgenes. This could be achieved either by the use of adenoviral vectors or by episomal vectors encoding the reprogramming factors [8]. The latter technique is based on episomes that maintain themselves through the Epstein-Barr nuclear antigen that drives replication of the episomes from an origin of replication. During passage cells tend to lose the episomes in an uncontrolled fashion if selection pressure is absent. In addition methods to utilize transposons were described [9, 10]. These techniques aim to remove the transgenes used for reprogramming when the process is completed but bear the risk of inducing changes to the genomic DNA like induction of frameshifts. Recently it could be shown that cell permeable reprogramming factors can perform full re-

programming of somatic cells when added as recombinant proteins to the culture medium [11].

The ability to derive pluripotent cells from each individual paves the way to generate patient-tailored iPS cells for therapeutical applications in the future. One essential prerequisite for these therapies to become reality is to insure that specific iPS cell-derived mature cells used for tissue repair can be obtained in sufficient amounts, and that they possess all required functional characteristics and are safe alternatives to ES- and adult-cell-based therapies.

The differentiation of murine iPS cells into CMs has recently been reported providing limited information about single or integrated cell electrophysiology [12-14]. Also Schenke-Layland and coworkers proved that iPS cells have the capability to differentiate *in vitro* into cardiovascular and hematopoietic lineages. Although they nicely showed typical Ca^{2+} -oscillations in iPS derived CMs [14], detailed information about electrophysiological characteristics of iPS CMs such as ion channel expression as well as their capability to generate action potentials and force is still lacking. Therefore the present study focussed on the electrophysiological characterisation of iPS CMs as compared with ES-CMs. We demonstrate that the cardiogenic potential of murine Oct4- and Nanog-selected iPS cells is similar to that of murine ES cells and that iPS cell-derived CMs possess typical functional and electrophysiological features of ES-CMs. Our data support the assumption that iPS cell-derived CMs may represent a valuable source of cells for cellular replacement strategies.

Materials and Methods

ES cell culture

Undifferentiated Oct4- or Nanog-selected iPS cell lines O9 and N10 were generated from murine embryonic fibroblasts (MEFs) by Wernig and coworkers [4]. For comparison with iPS cells conventional murine ES cell lines R1 [15] and D3 (clone α PIG44) [16] were used. The α PIG44 clone was chosen as a control because it is well characterized in terms of cardiac differentiation [16]. Culture of undifferentiated cells was performed on embryonic feeder cells in presence of LIF. See online supplement for details "<http://www.uni-koeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>". According to the differences in culture conditions we defined the following developmental stages for iPS EBs: EDS: day 11-16, LDS: day 21-25, and the following for D3- α PIG44 EBs: EDS: day 9-12, LDS: day 16-19.

Cardiac differentiation of reprogrammed iPS cells

Cardiac differentiation of iPS cells was performed using a mass culture system for EB production (Figure 1, online data

Fig. 1. RT-PCR analysis at different developmental stages. a) Course of *T-bra*, *AFP*, *Flk-1*, *SMA*, α MHC, *MLC2v*, *Mlc2a*, *Foxa2*, *Sox17*, *Fgf5* and *NF-H* expression in D3- α PIG44, R1, O9 and N10 EBs during differentiation. GAPDH was used as the housekeeping gene. The expression of all genes indicated was also determined in MEFs that were used for generation of iPS cells. b) Transcript levels of major cardiac-specific genes in undifferentiated O9 cells (lane 1), O9-CMs (lane 2) and N10-CMs (lane 3) collected on day 16 of differentiation. Samples without reverse transcription were used as negative controls (lane 4-6) and did not show any amplification products.

supplement “<http://www.uni-koeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>”). Briefly, single cell suspensions from ES and iPS cells were agitated inside a cell culture incubator at a density of 2.5×10^5 cells/ml in propagation medium devoid of LIF for 2 days (ES) or 4 days (iPS), and subsequently diluted to 1000 EBs per dish and further agitated. Plating of the EBs was done at day 5 (ES) or day 7 (iPS) on gelatine coated dishes. See online supplement for further details.

RNA Isolation and RT-PCR

For reverse transcriptase polymerase chain reactions (RT-PCR), cells or EBs were lysed in Trizol Reagent (Invitrogen) to isolate total RNA followed by standard protocols for further processing [17]. Total RNA was DNase treated and transcribed into cDNA using random hexamers and Superscript II Reverse transcriptase (Invitrogen). PCR was performed using Jump Start Red Taq polymerase kit (Sigma Aldrich). See online supplement “<http://www.uni-koeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>” for additional information.

Immunocytochemistry

Beating areas of plated EBs were dissected, plated onto cover slips and fixated according to standard protocols (see online supplement for details “<http://www.uni-koeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>”). Briefly anti-sarcomeric- α -actinin, anti-connexin 43, anti-ANP, anti-vinculin and anti-cardiac troponinT were used while nuclei were stained with Hoechst 33432. Secondary detection was performed with anti-mouse-IgG1-AlexaFluor555 or anti-rabbit-IgG1-AlexaFluor647. After washing, samples were embedded in ProLong Gold Antifade Reagent and evaluated using a Zeiss Axiovert 200/ ApoTome and Zeiss Axiovision 4.5 software.

Multi-Electrode Array (MEA) measurements

After plating of microdissected beating areas on MEAs, extracellular recording was performed using the MEA data acquisition system (Multi Channel Systems, Reutlingen/Germany) as described [18]. Standard measurements were performed at 1-2 kHz (bandwidth 1-5 kHz) in Iscove’s modified

Fig. 2. Identification of cardiomyocytes and gap junctions in N10 EBs. a-c) Immunocytochemical staining for cardiac troponin T (green) in N10 (a), D3- α PIG44 (b) and R1 (c) EBs. d-h) Sarcomeric- α -actinin (green) in beating EBs of N10 (d), D3- α PIG44 (e) and R1 (f). Staining for ANP (red) in these cells did not show ANP expression. In control stainings ANP was strongly expressed in native mouse atrial (g) but not in mouse ventricular (h) cardiomyocytes (embryonic day 17.5, LDS). i) Double-staining of cells in the outgrowth from a N10 EB for vinculin (yellow) and connexin43 (red). j,k) High resolution images from N10 (j) and O9 (k) CMs inside a beating cluster. Note sarcomeric- α -actinin in green, nuclei in blue and the expression of connexin43 in red. l) Immunocytochemical staining from a typical suspension EB culture at day 12 of differentiation with anti-sarcomeric- α -actinin (green). m) High resolution multifocus image of beating clusters from N10 cells showing a group of CMs in the outgrowth of a beating cluster. Sarcomeric- α -actinin is stained with green and nuclei with blue. The picture combines several optical sections through the cell cluster to enhance the deep focus. Note the cross striation typical for cardiomyocytes. n) Staining of an O9 EB with anti-sarcomeric- α -actinin (green). o) Three dimensional reconstruction of the cell cluster shown in (m). A stack of 15 single images with a total Z dimension of 2.5 μ m was converted into a three dimensional view of the cell cluster (sarcomeric- α -actinin: red, nuclei: blue). Scale bars: a-n) 50 μ m o) 10 μ m.

Dulbecco's medium without fetal calf serum (FCS) [19-21]. During recordings, temperature was kept at 37°C. Data were analyzed off-line with a customized toolbox programmed with MATLAB 5.0 (The Mathworks, Natick, MA, USA) as described [22].

Action potential (AP) recordings

Intracellular recordings of APs were performed with sharp electrodes (30-50 M Ω when filled with 3 mol/l KCl) made of borosilicate glass capillaries (WPI, Sarasota, USA) as published recently [23]. Briefly, for signal amplification a SEC-10LX amplifier (npi electronic, Tamm, Germany) was used. Data were acquired with the Pulse software package (HEKA, Lambrecht/Pfalz, Germany) and analyzed offline with the Mini Analysis program version 6.0.3 (Synaptosoft, Fort Lee, USA). All measurements were performed in suspension EBs at 37°C in DMEM (concentrations of inorganic salts in mmol/l: CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.3, NaHCO₃ 44, NaCl 110, NaH₂PO₄ 0.9) without serum bubbled with 5% CO₂ and 95% O₂.

Force Measurements

Force measurements of iPS-CMs or ES-CMs were performed as described in detail recently (see online supplement for details "<http://www.uni-koeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>") [24]. Briefly, neonatal murine ventricles were cut with a vibratome generating 300 μ m slices and completely avitalized by simulated hypoxia and ischemia for 20 hours. Thereby the ventricular slices lost their contractile function and could be further used as a dead natural and soft cardiac matrix tissue for transplantation experiments and recording of contractile function mediated by iPS-CMs. One beating cluster was cocultured on a slice for 5-10 days before relative changes of force were determined. Therefore coculture preparations were mounted onto steel needles, connected to an isometric force transducer and field stimulated at 0.5-10 Hz when required. Signals were recorded and analyzed using the software DasyLab version 7.0 (National Instruments, Munich, Germany).

Substances

If not stated otherwise, all substances were obtained from Sigma-Aldrich Chemie GmbH, Germany. Although specific I_f blockers such as ivabradine are commercially available we decided to choose CsCl to block I_f since, at least in our hands, ivabradine failed to block I_f completely. CsCl-sensitive, time-dependent current is clearly inwardly directed. Since only inward current is able to depolarize the cell during the diastolic depolarization phase pacemaking currents must be inward currents in the diastolic range. Thus, application of CsCl may potentially be better than the not completely blocking compound ivabradine to determine the contribution of I_f to spontaneous cardiac activity.

Data analysis

If not otherwise stated results are given as mean $\pm$ SEM for the number of cells indicated (n). Either paired or unpaired t-tests or one-way analysis of variance (ANOVA) was performed. Statistical significance was accepted when $p < 0.05$. Conduction velocities were calculated by dividing the distance between individual electrodes by the delay of the respective electrode-related field potential (FP).

Results

EB formation and cardiac differentiation

As a first step the appearance of beating areas during the differentiation of iPS cells and D3- α PIG44 ES cells were compared. During the optimisation of the differentiation protocol it turned out that the iPS cells required slight modifications in the protocol to form beating areas at an efficient rate. For details about the differentiation see the material and methods section. Upon optimisation of the cell culture conditions, the frequency of EBs containing spontaneously contracting CMs as determined at the first onset of beating in O9 ($43 \pm 15\%$) and N10 ($40 \pm 11\%$) cultures was comparable to that of usual D3- α PIG44 ($47 \pm 17\%$) differentiation (267 O9 and 369 N10 EBs were scored in three and 2753 D3- α PIG44 EBs in six independent differentiations).

Gene expression analysis

To characterize the differentiation potential of iPS cells towards the cardiac lineage in more detail, we analyzed mRNA expression profiles of several differentiation specific markers (Fig. 1a). The pluripotency marker *pou5f1* (*oct4*-coding gene) was expressed in O9 and N10 iPS cells, confirming the presence of undifferentiated cells (Fig. 1b). The abundance of the mRNAs for the t-box transcription factors Tbx2, Tbx3, Tbx5 and Tbx20 as well as the transcription factors islet-1 (Isl1), GATA binding proteins 4 and 6 (GATA 4 and GATA 6), which

are all involved in cardiac differentiation, were analysed. Furthermore several mRNAs coding for structural and functional proteins expressed in mature cardiomyocytes such as alpha myosin heavy chain (α MHC) being present in atrial and ventricular tissue, myosin light chain 2 ventricular (MLC2v) being mainly expressed in ventricular tissue, slow skeletal troponin I (ssTnI) and cardiac troponin T (cTnT) as Ca^{2+} binding cardiac specific proteins and ryanodine receptor 2 (RyR2) as cardiac sarcoplasmic reticulum associated Ca^{2+} release channel complex were studied. All these mRNAs were not present in undifferentiated O9 iPS cells (Fig. 1b) but were strongly expressed in beating cardiac O9 and N10 EBs at day 16 (Fig. 1b).

During differentiation the early mesodermal marker T-brachyury (T-bra) came up on day 5 in O9 and N10 EBs. Similarly, T-bra was expressed from day 4 on in the D3- α PIG44 and R1 ES cell-derived EBs. The cardiac marker genes α MHC and MLC2v were both expressed by iPS cell-derived EBs. Onset of the expression was dependent on the gene analysed and varied between ES and iPS cell lines studied. The atrial marker myosin light chain 2 atrial (Mlc2a) was weakly expressed in differentiating D3- α PIG44 and iPS cells and was not detectable in R1 EBs. Expression of the vascular endothelial growth factor receptor 2 (Flk-1) and smooth muscle actin (SMA) suggests formation of endothelial cells and smooth muscle cells and the presence of mRNA for forkhead box A2 (Foxa2), SRY box containing gene 17 (Sox17) and alpha fetoprotein (AFP) indicates the presence of endodermal cell lineages (Fig. 1a). Cells of the ectodermal germ layer were also formed in all cell lines tested as indicated by fibroblast growth factor 5 (FGF5) expression and by the expression of the neuronal marker gene neurofilament heavy (NF-H). With the exception of SMA, none of the genes tested were expressed in murine embryonic fibroblasts (Fig. 1a).

Taken together, developmental gene expression analysis indicates that iPS cells differentiated towards derivatives of all three germ layers including CMs. Although the expression of some differentiation markers appeared to be somewhat delayed in iPS cell lines, it remains to be determined in further studies whether this represents an iPS cell-specific phenomenon.

Detection of cardiac-specific proteins in iPS-CMs

As a further proof for cardiac differentiation we tested for the presence of cardiac cells in the three-dimensional EBs by staining for cardiac specific proteins. Figure 2a-c shows representative stainings for cardiac

Fig. 3. Intact functional expression of β -adrenergic signalling. a) Statistical analysis of Iso effects (1 μ M) in EDS N10 CMs (n=11) and EDS D3- α PIG44 CMs (n=3) as compared to controls. Data were significantly different between controls (p=0.002) and after Iso application (p=0.004). b) Analysis of Iso effects (1 μ M) on LDS N10 (n=5) and LDS D3- α PIG44 cardiac clusters (n=7) as compared to controls. The effects of Iso were significantly different between controls (p=0.017) and after Iso application (p=0.008).

Fig. 4. Intact muscarinic response. Effects of CCh were measured as modulation of spontaneous field potential frequency using cardiac clusters plated on MEAs. CCh (1 μ M) led to a significant decrease of beating frequency in N10-CMs at EDS (a, n=6, p<0.001) and N10-CMs at LDS (b, n=7, p=0.001). The effect of CCh was significantly stronger at LDS (p<0.001). For comparison effects of CCh (10 μ M) were studied in α PIG44-CMs at EDS (a, n=5) and LDS (b, n=8). The effect in α PIG44-CMs was significantly stronger at LDS (p<0.05) as compared to EDS.

TroponinT. High resolution images of these stainings revealed typical patterns of cross striation indicative of cardiac Troponin T (online suppl. Fig. IX, “<http://www.unikoeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>”). As in murine ES cell-derived EBs, CMs in spontaneously beating iPS EBs were found to be mainly arranged in clusters. Additional stainings for the cardiac specific sarcomeric alpha-actinin were performed in N10 and O9 iPS cell- and R1 and D3- α PIG44-derived CMs and demonstrated typical and similar striation patterns (Fig. 2d-f, green).

Stainings for the atrial marker atrial natriuretic peptide (ANP) were negative in D3- α PIG44 and iPS-de-

Concentration (mM)	Frequency (Hz)	SEM	n
Control	1.229	0.1985	5
0.001	1.246	0.1698	5
0.01	1.468*	0.2428	5
0.1	2.058*	0.1829	5
1.0	2.310*	0.2774	5
10.0	2.325*	0.2734	5
100.0	2.416*	0.1644	5

Table 1. Concentration-response of Iso on beating frequency during MEA measurements as compared to controls. *indicate statistical significance.

rived clusters (Fig. 2d-f, red), whereas intense ANP staining was observed in atrial (Fig. 2g) but not in ventricular (Fig. 2h) CMs isolated from murine E17.5 fetuses taken as controls.

Strong expression of connexin43, a gap junction protein expressed in CMs, was found in iPS cell-derived cardiac clusters (Fig. 2j, k). Presence of connexin43 expression at the cell/cell contacts in N10 EBs was demonstrated by double staining for connexin43 and vinculin (Fig. 2i). Staining of whole-mount EBs for sarcomeric α -actinin in N10 EBs underlines the localization of CMs in clusters (Fig. 2l) and high-resolution imaging showed typical cross-striation in N10 and O9 cardiomyocytes (Fig. 2m-o). In summary, all tested proteins show a similar expression pattern in D3- α PIG44 and iPS-derived cardiac clusters.

MEA recordings

A) Basic FP parameters. During the course of differentiation iPS-CMs developed higher beating frequencies (early developmental stage (EDS): 1.35 ± 0.06 Hz; late developmental stage (LDS): 2.22 ± 0.21 Hz, $p < 0.001$) and a shorter field potential (FP) duration (FPdur, EDS: 247.6 ± 15.9 ms; LDS: 131.8 ± 15.1 ms, $p < 0.001$). D3- α PIG44-CMs showed significantly faster beating frequencies when being compared with iPS cells but no significant differences when EDS and LDS derived D3- α PIG44-CMs were compared (EDS: 2.95 ± 0.24 Hz; LDS: 2.93 ± 0.23 Hz, $p > 0.05$; $p < 0.001$ for EDS D3- α PIG44-CMs vs. EDS iPS-CMs and $p = 0.04$ for LDS D3- α PIG44-CMs vs. LDS iPS-CMs). Likewise, we did not find significant differences when FPdur of EDS and LDS D3- α PIG44-CMs was compared. However, the FPdur was significantly shorter in D3- α PIG44-CMs than in iPS-CMs (EDS: 67.2 ± 4.6 ms, $p < 0.001$; LDS: 58.8 ± 4.1 ms, $p < 0.001$).

B) Effect of β -adrenergic agonists. To test for the response of iPS-CMs to hormonal stimulation, we applied isoproterenol (Iso) as a standard agonist of the β -adrenergic signalling cascade to cardiac clusters derived from EDS and LDS iPS and D3- α PIG44 EBs. Positive chronotropy was proven by extracellular FP frequency changes. The dose-response curve showed a typical sigmoid shape. The EC₅₀ value (50 nM) and Hill-coefficient (0.071 ± 0.064 , $n = 5$) of the Iso effect were comparable to those reported for murine and human ES-CMs [18]. Raw data and statistical analyses are shown in Table 1.

To test whether maturation changes the β -adrenergic response we applied 1 μ M Iso to N10 EDS (Fig. 3a)

and LDS (Fig. 3b) CMs. Addition of Iso resulted in a significant increase of FP frequency at both developmental stages. There was a trend ($p = 0.11$) to stronger effects of Iso at LDS ($+74.5 \pm 24.1\%$, $n = 5$) than at EDS ($+38.7 \pm 8.7\%$, $n = 11$) but the difference did not reach statistical significance. Application of 1 μ M Iso to EDS and LDS D3- α PIG44-CMs led to similar responses. Effects of Iso were statistically different between EDS ($p = 0.019$) but not between LDS ($p = 0.14$) N10 and D3- α PIG44-CMs.

C) Conduction velocity. To test for functional coupling of cardiomyocytes present in cardiac clusters, conduction velocity of the excitation spread was determined. The conduction velocities amounted to 0.24 ± 0.1 m/sec in N10 cardiac clusters ($n = 4$) and to 0.29 ± 0.24 m/sec in D3- α PIG44 ES-CMs ($n = 4$, $p > 0.05$). As a functional test for gap junction protein expression, the gap junction blocker heptanol (0.25 mM) was applied [25, 26]. Heptanol reduced conduction velocities to 0.09 ± 0.04 m/sec in N10 ($n = 4$, $p < 0.05$) and similarly to 0.04 ± 0.03 m/sec in D3- α PIG44 ES-CMs ($n = 4$, $p < 0.05$). Due to possible unspecific effects of heptanol we applied 40 μ M 18 α -Glycyrrhetic acid (AGA), another gap junction blocker in another set of experiments. Similar to heptanol, AGA reduced conduction velocities from 0.37 ± 0.22 m/sec to 0.14 ± 0.09 m/sec in N10 ($n = 4$) and from 0.49 ± 0.2 m/sec to 0.15 ± 0.12 m/sec in D3- α PIG44 ES-CMs ($n = 4$, $p < 0.05$).

D) Effects of muscarinic receptor agonists. To prove the presence of muscarinic signalling, carbachol (CCh), a synthetic acetylcholine analogon, was applied (Fig. 4). In N10 EDS-CMs and D3- α PIG44 EDS-CMs CCh evoked a significant reduction of the FP frequency to $84.5 \pm 2.6\%$ of controls in N10 ($n = 6$, $p < 0.001$) and $78.2 \pm 5.1\%$ ($n = 5$, $p < 0.05$) in D3- α PIG44-CMs (Fig. 4a).

Since previous studies suggested significant changes of muscarinic signalling during embryonic heart development, we tested CCh also in LDS-derived cardiomyocytes. Compared to EDS even stronger effects were observed at N10 LDS-CMs and D3- α PIG44 LDS-CMs (Fig. 4b), where CCh led to a reduction of the beating frequency to $39.8 \pm 8.8\%$ of controls in N10 ($n = 7$, $p = 0.001$) and $27.5 \pm 12.6\%$ ($n = 8$, $p < 0.05$) in D3- α PIG44-CMs. The effect of CCh was statistically significantly different between EDS and LDS ($p < 0.05$) in both cell lines, but there was no significant difference between D3- α PIG44 and N10 CMs.

E) Na^+ -channels, I_f , NCX and L-type Ca^{2+} channels are present in iPS-CMs and involved in controlling spontaneous beating frequency. Both, ES-CMs and iPS-CMs, are characterized by their spontaneous

Fig. 5. I_{Na^+} , I_{P} , I_{NCX} and I_{CaL} are functionally expressed and involved in spontaneous beating. Effects of lidocaine, CsCl, SEA0400, and verapamil+nifedipine on spontaneous beating of EDS (a) and LDS (b) N10-derived beating clusters in comparison to EDS (c) and LDS (d) α PIG44-derived beating clusters. As read out the extracellular FP frequency of spontaneously beating cardiac clusters was measured with MEAs. Drug effects are expressed as percent of inhibition of spontaneous beating.

beating. To obtain an idea which ion channels are generally involved in this autonomous beating, and whether there might be differences between iPS and ES cell-derived cardiomyocytes, different ion channel blockers were applied during MEA measurements.

In N10 and D3- α PIG44-CMs (EDS and LDS) we tested the effects of (i) lidocaine as a Na^+ -channel blocker, (ii) CsCl as I_f blocker, (iii) SEA0400 as NCX-blocker, and (iv) verapamil+nifedipine as L-type Ca^{2+} channel blockers. In general, all substances led to a significant negative chronotropy in N10 and D3- α PIG44-derived CMs suggesting their involvement in spontaneous electrical activity. Verapamil+nifedipine, in combination completely blocking L-type Ca^{2+} channel current (data not shown), led to a complete halt of spontaneous beating at EDS (N10: Fig. 5a, $n=8$; D3- α PIG44: Fig. 5c, $n=5$) and LDS (N10: Fig. 5b, $n=6$; D3- α PIG44: Fig. 5d, $n=4$) (see also Fig. II-V, online data supplement “<http://www.uni-koeln.de/80/med-fak/physiologie/np/pfann/pfann.htm>”). In contrast, the other ion channel blockers only led to a moderate decrease of spontaneous beating.

In N10, lidocaine significantly decreased frequency to $85.7 \pm 9.0\%$ of controls at EDS ($n=4$, $p=0.047$) and to

$59.0 \pm 3.7\%$ at LDS ($n=6$, $p=0.001$), whereas in D3- α PIG44-derived CMs a reduction to $65.2 \pm 5.0\%$ of controls at EDS ($n=3$, $p=0.046$) and to $37.9 \pm 7.3\%$ at LDS ($n=4$, $p=0.004$) was found. CsCl led to a significant reduction of FP frequency at EDS (N10: to $66.6 \pm 5.2\%$, $n=6$, $p=0.004$; D3- α PIG44: to $51.4 \pm 8.3\%$, $n=6$, $p=0.035$) and at LDS (N10: to $86.6 \pm 4\%$, $n=8$, $p=0.02$; D3- α PIG44: to $70.3 \pm 9.9\%$, $n=6$, $p=0.036$).

SEA0400 led to a reduction of FP frequency at EDS (N10: to $26.8 \pm 8.8\%$, $n=7$, $p=0.02$; D3- α PIG44: to $42.7 \pm 8.4\%$, $n=5$, $p=0.037$) and LDS (N10: to $58.2 \pm 6.5\%$, $n=6$, $p=0.007$; D3- α PIG44: to $67.5 \pm 9.9\%$, $n=5$, $p=0.048$). Taken together the ion channel blockers led to similar responses in iPS and ES cell-derived cardiomyocytes and showed different effects at later stages of cardiac development.

Action potential measurements

To elucidate whether EBs differentiate towards atrial and/or ventricular CMs and to test iPS-CMs and D3- α PIG44 ES-CMs more specifically for intact cellular electrophysiology, spontaneous action potentials (APs) were recorded. Fig. 6a shows representative APs re-

Fig. 6. iPS-CMs mainly differentiate towards ventricular-like cells. a) Representative APs recorded in single cardiomyocytes dissociated from EDS (upper left panel) and LDS (upper right panel) N10 ES-CMs. Note the increase of AP amplitude and shortening of the APD at LDS. The lower panel highlights the different AP morphologies that were recorded at LDS. These APs suggest a differentiation towards ventricular-like cells. b) APD was found to be dependent on beating rate suggesting intact cardiac physiology. c) Pharmacological response. Iso (1 μ M) significantly increased the AP frequency and APD90. CsCl led to a significant decrease of AP frequency and intermittent occurring bursting of APs. Nifedipine completely blocked spontaneous beating. During begin of washout the strong effect of L-type Ca^{2+} channel blockade on the AP morphology became evident. Note the strong reduction of APD and AP amplitude. Blocking NCX also strongly reduced the AP frequency and APD90 suggesting that NCX contributes to the plateau phase (see also Table 3-6, online data supplement). Washout for SEA0400 was only possible after hours.

corded from single cardiomyocytes dissociated from EDS and LDS, suggesting a high rate of ventricular differentiation in iPS EBs. D3- α PIG44 however, are known to differentiate mainly towards atrial-like CMs. Accordingly significant differences between iPS-CMs and D3- α PIG44-CMs were found for AP frequency, APD₅₀, APD₉₀ and AP amplitude Table 7.

As supporting proof for intact cardiac electrophysiology, APD was found to be dependent on the beating rate (Fig. 6b). In accordance with our MEA data more detailed correlation analyses of AP parameters revealed similar results for iPS-CMs and ES-CMs. These data suggest a full expression of the main cardiac sarcolemmal ion channels (supplemental Fig. VI, “<http://www.uni-koeln.de/80/med-fak/physiologie/np/pfann/pfann.htm>”). Since the MEA data also point to a maturation of the iPS-CMs, we performed further statistical analysis that revealed typical AP parameter changes during

Parameter	EDS (n=9)	LDS (n=16)	p
MDP (mV)	-47.8 $\pm$ 3.5	-63.4 $\pm$ 1.5	<0.0001
Overshoot (mV)	5.9 $\pm$ 1.2	11.8 $\pm$ 1.9	0.036
Vmax (V/s)	11.7 $\pm$ 1.7	27.7 $\pm$ 3.5	0.003
APD50 (ms)	78.4 $\pm$ 5.7	64.2 $\pm$ 3.5	0.032
APD90 (ms)	122.3 $\pm$ 6.2	26.3 $\pm$ 0.1	<0.0001
Frequency (Hz)	1.3 $\pm$ 0.1	2.0 $\pm$ 0.2	0.056
Amplitude (mV)	53.7 $\pm$ 3.7	75.3 $\pm$ 2.2	<0.0001

Table 2. AP parameters of EDS and LDS iPS-CMs.

development (Table 2).

Another prerequisite for intact cardiac electrophysiology is the coupling of sarcolemmal ion channels to the cytosolic signalling cascades that subsequently lead to changes of AP parameters. Stimulation of the β -adrenergic signalling cascade by Iso evoked a typical signifi-

Parameter	LDS control (n=3)	LDS Iso (n=3)	p
MDP (mV)	-65.00±2.31	-59.33±7.22	0.414
Overshoot(MV)	6.00±3.00	15.33±6.01	0.224
Vmax (V/s)	26.74±4.34	27.17±1.79	0.927
APD50 (ms)	24.67±6.82	24.84±4.33	0.950
APD90 (ms)	57.94±5.00	53.17±5.04	0.023
Frequency (Hz)	1.95±0.43	3.41±0.47	0.003
Amplitude (mV)	71.00±3.46	74.67±1.54	0.599

Table 3. Effects of Iso on AP parameters.

cant increase of the AP frequency (Fig. 6c, upper panel) in iPS-CMs, and a significant decrease of the APD90 (Table 3).

CsCl as I_T blocker significantly decreased AP frequency (Table 4). Consistent with findings in early murine ES-CMs, we found a typical bursting of APs after initial hyperpolarization of the membrane potential (Fig. 6c), suggesting involvement of ATP-dependent K^+ -channels [27]. In LDS D3- α PIG44-CMs negative chronotropy without AP bursting was observed (Figure VII “<http://www.uni-koeln.de:80/med-fak/physiologie/np/pfann/pfann.htm>”).

In line with our MEA data, 1 μ M nifedipine led to a complete halt of APs in both cell lines (Fig. 6c, Table 5). When being washed out, first membrane potential oscillations were observed followed by initiation of APs, before the APs were completely restored. The overlay of respective single APs showed the important influence of I_{CaL} on AP generation (Fig. 6c).

SEA0400 as NCX blocker led to a significant reduction of the AP frequency and shortening of APD90 (Fig. 6c, Table 6). These data suggest that besides L-type Ca^{2+} channels also NCX plays a major role for embryonic electrophysiology as recently reported by our group. Control measurements with several ion channel blockers in D3- α PIG44-CMs are shown in Table 8-11. In summary our data show similar results for iPS-CMs and ES-CMs.

Force measurements

Since contractility is the primary task of cardiomyocytes, we analyzed whether iPS-CMs and D3- α PIG44-CMs possess similar contractile properties (Fig. 7a) [28]. Avitalized myocardial control tissue slices did not develop force, not even under electrical stimulation (20V, 100 ms) (Fig. 7b, n=5). To generate avitalized tissue we applied oxygen and glucose deprivation as described in detail in the online supplementary method section. These soft avitalized tissues served as a physiologi-

Parameter	LDS control (n=3)	LDS CsCl (n=3)	p
MDP (mV)	-61.7±0.7	-63.7±3.5	0.58
Overshoot (mV)	12.7±8.2	7.3±3.7	0.58
Vmax (V/s)	17.0±3.8	19.5±3.0	0.44
APD50 (ms)	19.8±9.9	22.7±8.6	0.34
APD90 (ms)	63.7±14.6	76.1±7.5	0.47
Frequency (Hz)	3.7±0.1	2.7±0.2	0.033
Amplitude (mV)	74.3±7.7	74.7±1.5	0.973

Table 4. Effect of I_T blockage by CsCl on AP parameters.

Parameter	LDS control (n=3)	LDS Nifedipine (n=3)	p
MDP (mV)	-65.67±7.31	-59.0±5.7	0.63
Overshoot(MV)	15.7±5.7	-59.0±5.7	<0.0001
Vmax (V/s)	26.4±6.7	0	<0.0001
APD50 (ms)	17.0±1.2	0	<0.0001
APD90 (ms)	42.3±3.7	0	<0.0001
Frequency (Hz)	3.3±0.7	0	<0.0001
Amplitude (mV)	82.3±6.2	0	<0.0001

Table 5. Effects of L-type Ca^{2+} channel blockage on AP parameters.

Parameter	LDS control (n=3)	LDS SEA0400 (n=3)	p
MDP (mV)	-75.3±4.1	-61.0±6.1	0.25
Overshoot (mV)	8.0±7.5	4.0±5.0	0.26
Vmax (V/s)	51.7±19.6	32.5±1.6	0.46
APD50 (ms)	30.2±2.3	17.9±4.3	0.15
APD90 (ms)	51.2±3.8	31.1±4.3	0.021
Frequency (Hz)	4.4±0.4	2.1±0.3	0.012
Amplitude (mV)	83.8±8.7	65.0±6.3	0.15

Table 6. Effects of NCX forward and reverse mode blockage on AP parameters.

Parameter	iPS (n=16)	LDS ES (n=15)	p
MDP (mV)	-67.1±1.9	-66.0±1.9	0.782
Overshoot (mV)	13.3±2.6	4.0±1.6	0.110
Vmax (V/s)	29.8±3.9	21.6±4.7	0.233
APD50 (ms)	29.8±3.9	20.4±2.3	0.052
APD90 (ms)	61.3±4.6	43.4±2.7	0.003
Frequency (Hz)	3.0±0.3	5.3±0.5	0.001
Amplitude (mV)	80.4±3.0	70.4±2.2	0.019
APD50/APD90 (%)	47.2±4.2	45.7±2.5	0.758

Table 7. AP parameters of iPS versus ES-CMs.

cal matrix for iPS cell-derived CMs as it resembled the native matrix protein composition of the murine heart.

Parameter	LDS control (n=3)	LDS Iso (n=3)	p
MDP (mV)	-69.8±2.6	-70.0±2.1	0.95
Overshoot (mV)	3.7±5.5	3.7±6.5	1.0
Vmax (V/s)	11.6±5.0	16.4±2.9	0.19
APD50 (ms)	16.7±1.6	17.4±0.8	0.39
APD90 (ms)	39.0±6.7	40.6±6.3	0.77
Frequency (Hz)	6.30±1.2	3.42±0.76	0.011
Amplitude (mV)	73.5±9.7	73.7±9.3	0.74

Table 8. Effects of I_f blockage by 5mM CsCl on AP parameters in α PIG44-CMs.

When avitalized tissue sections were being ‘transplanted’ with beating cell clusters generated from iPS-EBs (Fig. 7c) and D3- α PIG44-EBs (Fig. 7d) we observed a spontaneous beating of the transplanted cells that were transferred to the matrix. When hooked up to a force transducer the generation of force during spontaneous beating and electrical field stimulation could be recorded. Although the developed force was higher in iPS (spontaneous: 1.35 ± 0.49 μ N, stimulated: 1.52 ± 0.46 μ N, n=5) than in ES-CMs preparations (spontaneous: 0.94 ± 0.14 μ N, stimulated: 1.03 ± 0.07 μ N, n=5), the difference did not reach significance (spontaneous: p=0.24, stimulated: p=0.22). Application of Iso (1 μ M) increased the force developed in iPS-CMs to $120\pm5\%$ (n=4) and in mES cells to $123\pm5\%$ of baseline (n=4, p=0.718, Fig. 7e).

In conclusion, iPS-CMs develop and confer force *in vitro*, show similar electrophysiological characteristics and exert similar pharmacological responses to β -adrenergic stimulation as CMs differentiated from blastocyst-derived ES cells.

Discussion

In this study we show that reprogrammed iPS cells selected either by Oct4 (O9 line) or Nanog (N10 line) expression efficiently differentiate towards cardiac muscle cells *in vitro*. The iPS cell-derived CMs exhibited (i) intact response to β -adrenergic and (ii) muscarinic agonists, (iii) expressed I_{Na} , I_P , I_{NCX} and I_{CaL} with I_{CaL} being of major importance, (iv) conferred force of contraction responding to Iso with positive inotropy, and (v) matured towards ventricular-like cells during differentiation up to day 23 *in vitro*.

Cellular cardiomyoplasty with ES cell-derived cells is hampered by rejection of immunologically mismatched grafts. Recent success in epigenetic reprogramming of

Parameter	LDS control (n=3)	LDS Iso (n=3)	p
MDP (mV)	-61.3±0.9	-61.0±10.8	0.97
Overshoot (mV)	-2.0±3.6	4±3.6	0.10
Vmax (V/s)	15.2±5.3	21±11.4	0.30
APD50 (ms)	14.7±2.5	15.5±3.7	0.41
APD90 (ms)	38.0±4.1	37.7±5.4	0.80
Frequency (Hz)	6.4±0.4	8.0±0.6	0.03
Amplitude (mV)	73.5±9.7	65.0±7.5	0.61

Table 9. Effects of 1 μ M Iso on AP parameters in α PIG44-CMs.

Parameter	LDS control (n=3)	LDS Lidocaine (n=3)	p
MDP (mV)	-63.3±5.4	-65.3±5.8	0.44
Overshoot (mV)	4.3±2.9	3.0±6.8	0.77
Vmax (V/s)	22.4±5.9	10.6±2.6	0.15
APD50 (ms)	14.3±1.1	19.0±2.8	0.19
APD90 (ms)	33.9±2.8	44.1±4.9	0.21
Frequency (Hz)	7.1±0.8	3.2±0.3	0.017
Amplitude (mV)	68.3±3.2	68.3±1.2	1.0

Table 10. Effects of Na^+ channel blockage on AP parameters.

Parameter	LDS control (n=3)	LDS Nifedipine (n=3)	p
MDP (mV)	-58.0±2.6	-55.7±3.8	0.073
Overshoot (mV)	3.0±3.6	-55.7±3.8	0.003
Vmax (V/s)	33.4±6.5	0.0±0.0	0.012
APD50 (ms)	14.9±1.4	0.0±0.0	0.003
APD90 (ms)	38.4±5.6	0.0±0.0	0.007
Frequency (Hz)	6.5±1.0	0.0±0.0	0.008
Amplitude (mV)	60.7±6.5	0.0±0.0	0.004

Table 11. Effects of L-type Ca^{2+} channel blockage on AP parameters.

somatic cells into pluripotent ES cell-like cells [4, 7, 29-32] offers new strategies for cellular replacement [33] as such cells would allow for autologous transplantation without rejection [34]. However, this potential will be realized only if reprogrammed cells, such as cardiomyocytes, [12] possess all functional characteristics of the host tissue.

Recently, Schenke-Layland and colleagues nicely described the differentiation of iPS cells into the cardiovascular lineage [12]. Detection of intracellular Ca^{2+} -transients pointed to an intact cellular physiology. Almost at the same time Narazaki and colleagues [14] published data confirming the findings of Schenke-Layland et al. They measured the generation of action potentials in beat-

Fig. 7. Force measurements after *in vitro* transplantation. a) Experimental protocol of transplantation. b) Recordings of avitalized ventricular tissue slices after simulated hypoxia and ischemia without transplanted cells (left), after co-culture with beating areas derived from iPS (N10) (middle) and ESCs (D3- α PIG44) (right) without (upper row) and during (lower row) electrical field stimulation (4 Hz). c) Comparison of force of co-cultures with N10 and D3- α PIG44-derived beating areas. d) Force measurements before (gray) and after application of 1 μ M Iso (black) during electrical field stimulation (4-6 Hz). e) Comparison of the effect of 1 μ M Iso on co-cultures with N10 and D3- α PIG44-derived beating areas. *indicates significant difference compared with controls or baseline measurements ($P < 0.05$).

ing iPS derived CMs and made use of immunocytochemical stainings to prove the abundance of cardiac troponin T, connexin 43, HCN4, Cav3.2 and Kir2.1 in the cytoplasm or at the cell membrane of iPS CMs. Another publication by Mauritz and colleagues demonstrated that the Oct4-Neo selected iPS cell line O9 can be differentiated into CMs *in vitro*. The resulting CMs expressed marker genes and immunocytochemical staining pattern typically of ES cell derived CMs. Also Mauritz et al. found intracellular Ca^{2+} -fluctuations and isoproterenol- and carbachol-induced modulation of contraction frequencies suggesting adrenergic and muscarinic receptor dependent regulation of O9-derived CMs. As a detailed electrophysiological characterization is of major importance to understand the potential of iPS cells we aimed to address the electrophysiological properties of iPS de-

rived CMs, that are in contrast to previous studies selected based on the reactivation of the endogenous *Nanog* gene, in more detail. As controls a series of experiments were also performed with O9 iPS cells.

Using an adapted differentiation system, we obtained efficient differentiation of iPS cells towards the cardiac lineage. Which was in agreement with our RT-PCR data showing that iPS derived EBs express several cardiac specific transcripts. The lack of ANP expression (data not shown) being confirmed by immunocytochemical stainings for ANP may reflect the low proportion of atrial-like cells in total EB cell mass or delayed appearance of matured atrial cells in the course of ES and iPS cell differentiation.

However, the presence of other cardiac structural and functional proteins indicate that iPS-CMs have the

morphological characteristics of ES-CMs. Sarcomeric α -actinin as well as cardiac troponin T stainings of iPS-CMs revealed the typical cross-striation known for differentiated murine [16] and human ES-CMs [35].

Connexin43 staining suggests that iPS-CMs are morphologically and functionally coupled as described for murine [36], rhesus monkey [37], and human [18] ES-CMs. AP measurements suggest that the predominant cells detected at differentiation stages up to day 23 are of ventricular-like type [38].

Functional analysis revealed intact β -adrenergic and muscarinic signalling pathways, representing main regulatory systems for cardiac contractility. In accordance with our previous findings in human and murine ES-CMs [18], iPS-CMs strongly responded to Iso with a similar EC50 value and Hill coefficient [18]. Also the effects of CCh were similar to those previously reported for ES cells [18].

Na⁺- and L-Type Ca²⁺-channels, funny current and NCX were substantially active in N10 iPS-CMs and D3- α PIG44 ES-CMs [39, 40]. Since the blockage of L-type Ca²⁺ channels led to a full halt of beating, we postulate its major importance for generation and uphold of spontaneous electrical activity. This is in line with previous reports demonstrating that intracellular rise of Ca²⁺ activates NCX, leading to subthreshold depolarizations of the membrane potential and/or APs [41].

Generation of contractile force is of pivotal importance in cardiac cells. Our results demonstrate that iPS derived CMs have similar but not identical functions as ES-derived CMs and can actively confer force to a physi-

ological substrate. Nevertheless Iso evoked a physiological response in ES and iPS-derived CMs, indicating a fully expressed β -adrenergic signaling cascade leading to typical positive inotropy.

Apart from all these similarities between iPS- and D3- α PIG44 ES-CMs, iPS-CMs showed a slower beating frequency and subsequently a significantly longer FPdur than D3- α PIG44-CMs. This finding can be explained by the observation that D3- α PIG44 ES-CMs differentiate mainly towards an atrial phenotype [16], whereas our findings suggest a high amount of ventricular directed differentiation of the iPS cell line. This might also explain why Iso showed stronger effects in D3- α PIG44 ES-CMs.

In summary, our findings indicate that reprogrammed murine cells can give rise to functional cardiomyocytes that are electrophysiologically intact and possess functional receptors and downstream signalling cascades. Therefore, iPS-CMs can be regarded as potentially valuable source of cells for cellular cardiomyoplasty.

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