Cardiomyocytes Obtained From Induced Pluripotent Stem Cells With Long-QT Syndrome 3 Recapitulate Typical Disease-Specific Features In Vitro

Daniela Malan,* Stephanie Friedrichs,* Bernd K. Fleischmann, Philipp Sasse

Rationale: Current approaches for the investigation of long-QT syndromes (LQTS) are mainly focused on identification of the mutation and its characterization in heterologous expression systems. However, it would be extremely helpful to be able to characterize the pathophysiological effects of mutations and to screen drugs in cardiomyocytes.

Objective: The aim of this study was to establish as a proof of principle the disease-specific cardiomyocytes from a mouse model with LQTS 3 by use of induced pluripotent stem (iPS) cells and to demonstrate that the mutant cardiomyocytes display the characteristic pathophysiological features in vitro.

Methods and Results: We generated disease-specific iPS cells from a mouse model with a human mutation of the cardiac Na⁺ channel that causes LQTS 3. The control and LQTS 3–specific iPS cell lines were pluripotent and could be differentiated into spontaneously beating cardiomyocytes. Patch-clamp measurements of LQTS 3–specific cardiomyocytes showed the biophysical effects of the mutation on the Na⁺ current, with faster recovery from inactivation and larger late currents than observed in controls. Moreover, LQTS 3–specific cardiomyocytes had prolonged action potential durations and early afterdepolarizations at low pacing rates, both of which are classic features of the LQTS 3 mutation.

Conclusions: We demonstrate that disease-specific iPS cell–derived cardiomyocytes from an LQTS 3 mouse model with a human mutation recapitulate the typical pathophysiological phenotype in vitro. Thus, this method is a powerful tool to investigate disease mechanisms in vitro and to perform patient-specific drug screening.

Key Words: cardiac electrophysiology • cell culture • induced pluripotent stem cells • long QT syndrome • SCN5A

Long-QT syndrome (LQTS) is a severe disorder of the electric activity of the heart. It is caused by delayed repolarization of cardiomyocytes, which leads to abnormally long action potential (AP) durations that result in a prolonged QT interval in the ECG.1 The lifespan of patients with LQTS is often limited because of the development of ventricular tachycardia and sudden cardiac death.1,2 Generally, LQTS are caused by loss-of-function mutations, but they can also be caused by gain-of-function mutations, the most common being LQTS 3. The most frequent LQTS 3 mutation is the deletion of the amino acids lysine-proline-glutamine (ΔKPQ) in the intracellular loop between domains III and IV of the cardiac Na⁺ channel.1,3 This results in reactivation of Na⁺ channels during the late phase of the AP, which leads to prolongation of the AP duration and QT interval, as well as dangerous early afterdepolarizations (EADs) at slow heart rates.3 Because of the strong frequency dependence of this effect, patients with LQTS 3 have lethal arrhythmias preferentially at rest and during sleep.1,2

The common pathophysiological feature of LQTS is the prolonged AP duration in cardiomyocytes. This cannot be investigated directly in vitro because sufficient numbers of human ventricular cardiomyocytes cannot be harvested from patients. Therefore, the properties of mutated ion channels are preferentially investigated in heterologous expression systems that lack the typical cell biological and physiological features of cardiomyocytes.2,4

Induced pluripotent stem (iPS) cells can be propagated to an unlimited extent and have been shown to serve as a source of cardiomyocytes in vitro.5 Moreover, it has been shown recently that iPS cells derived from patients with LQTS 1 or 2 can be used to obtain disease-specific cardiomyocytes.5,7 In the present study, we provide evidence that cardiomyocytes differentiated from disease-specific iPS cells do recapitulate the typical frequency-dependent features of the LQTS 3 phenotype in vitro.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

iPS cells were generated by retroviral transduction of murine embryonic fibroblasts (MEFs) from Scn5aΔKPQ/+ mice (mice heterozy-
gous for the KPQ deletion of Scn5a) and wild-type littermates either with the 3 factors Oct4, Sox2, and Klf4 or additionally with the fourth factor c-Myc, as described previously.\textsuperscript{8,9} Teratoma assay was performed by injection of undifferentiated iPS cells into SCID mice. For in vitro differentiation of iPS cells, the hanging drop method was used to generate embryoid bodies as described previously.\textsuperscript{10} Immunostainings were performed according to standard protocols,\textsuperscript{10} and TaqMan assays (Applied Biosystems, Foster City, CA) were used for quantitative polymerase chain reaction.

Results

Generation of Disease-Specific iPS Cell Lines

iPS cells were generated from MEFs derived from wild-type and Scn5a\textsuperscript{+/+} littermates. For this purpose, MEFs were prepared from single embryos, genotyped (Figure 1B), and infected with retroviruses to express specific “stemness” factors.\textsuperscript{8} Reprogramming efficiency was enhanced by the addition of extracellular signal-regulated kinase and glycogen synthase kinase 3 inhibitors.\textsuperscript{9} Embryonic stem (ES) cell–like colonies appeared after 14 days and were selected on the basis of their ES cell–like morphology. Wild-type and Scn5a\textsuperscript{+/+} iPS cell lines were propagated on irradiated MEF layers and retained the morphology of undifferentiated ES cells (Figure 1A). Both wild-type and Scn5a\textsuperscript{+/+} iPS cells expressed the stem cell markers Oct4 and SSEA1 (Figure 1C). As expected,

\[ \text{Na}^+ \text{ currents were recorded and biophysically characterized from differentiated cardiomyocytes by use of the patch-clamp technique as described previously.}^{3,4,10,11} \text{ For recording of frequency-dependent AP durations, APs were evoked at various pacing periods, and the slope of the AP duration at 90\% of repolarization (APD}_{90}–pacing period relationship was analyzed for each individual cell by a linear regression.} \]
genotyping of iPS cell lines revealed the mutation in the Scn5aΔ/+ but not in the wild-type iPS cells (Figure 1B).

**Pluripotency of Wild-Type and Scn5aΔ/+ iPS Cells**

The pluripotency of established iPS cell lines was proven by in vivo teratoma formation and in vitro differentiation. After injection of wild-type and Scn5aΔ/+ iPS cells into SCID mice, teratoma developed with tissues from all 3 germ layers (Figure 1D). We also analyzed the in vitro differentiation characteristics and stained embryoid bodies at day 10 for markers of cells from the 3 different germ layers. Both wild-type and Scn5aΔ/+ iPS cell lines showed Troma-1–positive cells, which indicates endodermal differentiation (Figure 2A). Mesodermal differentiation (Figure 2A) was proven on the basis of platelet endothelial cell adhesion molecule-1–positive endothelial cells and α-actinin–positive cardiomyocytes. The presence of nestin- and βIII-tubulin–positive cells highlighted ectodermal differentiation (Figure 2A). Wild-type and Scn5aΔ/+ embryoid bodies showed typical spontaneous beating areas starting around day 7 of differentiation (supplementary Videos I and II). Relative gene expression analysis by quantitative polymerase chain reaction revealed a similar expression of stem cell markers (Oct4 and Nanog) in wild-type and Scn5aΔ/+ iPS cells that decreased on differentiation (Figure 2B). Expression of the cardiac-specific markers α-myosin heavy chain (Myh6), Na,1.5 Na⁺ channel (Scn5a), hyperpolarization-activated cyclic nucleotide-gated channel 4 (Hcn4), K,2,1 delayed rectifier K⁺ channel (Kcnb1), Ca,1.2 L-type Ca²⁺ channel (Cacna1c), and α-actinin (Acnt2) increased to a similar extent during differentiation of wild-type and Scn5aΔ/+ iPS cells (Figure 2C). Interestingly, expression of the Na⁺ channel (Scn5a) further increased with ongoing differentiation from day 9 to day 14.

**Biophysical Characterization of Na⁺ Currents in Disease-Specific and Wild-Type Cardiomyocytes From iPS Cells**

To measure Na⁺ currents and APs in single wild-type and Scn5aΔ/+ iPS cell-derived cardiomyocytes, single cells were isolated from beating areas of embryoid bodies and investigated by use of the patch-clamp technique. Cardiomyocytes from both lines were spontaneously beating and showed similar Na⁺ channel distribution and well-organized sarcomeric structures with Scn5a and α-actinin staining (Figure 3A).

Because LQTS 3 is caused by mutated Na⁺ channels, we investigated the functional expression of the voltage-dependent Na⁺ current at early (day 12) and late (late developmental stage, days 19 to 22) stages of differentiation using voltage ramps (data not shown). The percentage of cells with Na⁺ currents was lower in the early developmental stage (66.6% of wild-type [n=12] and 66.6% of Scn5aΔ/+ [n=15] cells) than in the late developmental stage (92.8% of wild-
and 76.4% of Scn5a+/cells). This is in line with gene expression data of the Na\(^+\) channel (Figure 2C), and therefore, only cardiomyocytes from the late developmental stage were investigated further. The capacitance of these cells was very similar (Table 1), which excludes the possibility that functional differences were related to cell size. Na\(^+\) current density at 10 mV was similar in wild-type and Scn5a+/cells (Figures 3B and 3C; Table 1). We also did not observe differences between genotypes in the steady state activation (ss\(_{\text{act}}\)) and inactivation (ss\(_{\text{inact}}\)) curves for wild-type and Scn5aΔ/+ cardiomyocytes (D) and statistical analysis of the potential of half-maximal (Half-max) activation and inactivation (E). F, Analysis of recovery from inactivation in a representative wild-type and Scn5aΔ/+ cardiomyocyte (monoeponential fit shown by dashed lines). G, Statistical analysis of the time constants of recovery from inactivation. H, Representative traces of late Na\(^+\) current before and after tetrodotoxin (TTX) application (20 \(\mu\)mol/L) in a wild-type (top) and an Scn5aΔ/+ (lower) cardiomyocyte. I, Statistical analysis of TTX-sensitive late Na\(^+\) current densities. Nuclei are shown in blue. Scale bar, 20 \(\mu\)m. Dotted lines indicate zero current; error bars, SEM. *P<0.05.

Characterization of APs From Wild-Type and Scn5aΔ/+ iPS Cell-Derived Cardiomyocytes

Because prolonged duration of APs, especially at low frequencies, is the typical hallmark of LQTS 3, we evoked APs in the current clamp mode and focused primarily on AP duration at different pacing frequencies. Amplitude, upstroke velocity, and resting membrane potential were similar in both genotypes (Table 2); however, clear differences were observed when we analyzed frequency-dependent AP durations. Wild-type cardiomyocytes had similar AP durations at fast and slow pacing rates (Figure 4A). In contrast, Scn5aΔ/+ cardiomyocytes showed prolonged AP durations when paced at slower rates (Figure 4B).

Because of the high variability of AP duration at APD\(_{90}\) between individual cells, only a tendency toward prolonga-
tion of averaged APD$_{90}$ was noted in Scn5aΔ/+ cells (Figure 4C). Because of the cell-to-cell variability, we quantified the dependency of AP duration of pacing periods (APD$_{90}$–pacing period) in individual cardiomyocytes using linear regression analysis (examples in Figure 4D). In wild-type cells, APD$_{90}$ was hardly affected by pacing frequency, therefore yielding a flat slope in the APD$_{90}$–pacing period relationship (Figure 4E; Table 2). In contrast, this was significantly different in Scn5aΔ/+ cardiomyocytes, in which the slope was steeply positive (Figure 4E; Table 2). In addition, approximately half of the Scn5aΔ/+ cardiomyocytes developed EADs at low pacing rates (Figures 4F and 4G), which were not observed in any of the wild-type cells (Figures 4F and 4G).

**Table 1. Biophysical Parameters of Na⁺ Currents**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>Scn5aΔ/+</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance, pF</td>
<td>54.6 ± 8.4, n = 33</td>
<td>49.8 ± 7.1, n = 27</td>
<td>0.66</td>
</tr>
<tr>
<td>$i_{\text{Na}}$ peak, pA/pF</td>
<td>23.3 ± 4.4, n = 9</td>
<td>21.2 ± 4.6, n = 7</td>
<td>0.75</td>
</tr>
<tr>
<td>SS$_{\text{act}}$ V1/2, mV</td>
<td>−44.3 ± 2.4, n = 15</td>
<td>−41.3 ± 1.0, n = 7</td>
<td>0.26</td>
</tr>
<tr>
<td>SS$_{\text{inact}}$ V1/2, mV</td>
<td>−67.8 ± 3.5, n = 12</td>
<td>−62.6 ± 3.0, n = 11</td>
<td>0.27</td>
</tr>
<tr>
<td>$\tau$ Deact − 40 mV, ms</td>
<td>3.63 ± 0.95, n = 14</td>
<td>3.47 ± 1.17, n = 8</td>
<td>0.92</td>
</tr>
<tr>
<td>$\tau$ Deact 0 mV, ms</td>
<td>2.43 ± 0.60, n = 13</td>
<td>2.07 ± 0.72, n = 6</td>
<td>0.71</td>
</tr>
<tr>
<td>$\tau$ Recovery, ms</td>
<td>8.23 ± 1.67, n = 7</td>
<td>3.90 ± 0.96, n = 7</td>
<td>0.0496</td>
</tr>
<tr>
<td>$i_{\text{Na}}$ late, pA/pF</td>
<td>1.38 ± 0.56, n = 7</td>
<td>3.86 ± 0.67, n = 8</td>
<td>0.0144</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. $i_{\text{Na}}$ peak indicates Na⁺ current density at −10 mV; SS$_{\text{act}}$, half-maximal voltage of steady state activation; SS$_{\text{inact}}$, half-maximal voltage of steady state inactivation; $\tau$ Deact, time constant of the kinetic of deactivation at −40 mV and 0 mV; $\tau$ Recovery, time constant of recovery from inactivation; and $i_{\text{Na}}$ late, current density of tetrodotoxin-sensitive late Na⁺ current. $P$ determined by Student $t$ test.

**Table 2. AP Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>Scn5aΔ/+</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP at 1 Hz, mV</td>
<td>−80.0 ± 3.5, n = 6</td>
<td>−83.0 ± 3.3, n = 7</td>
<td>0.55</td>
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<tr>
<td>APA at 1 Hz, mV</td>
<td>96.7 ± 4.9, n = 6</td>
<td>110.0 ± 3.8, n = 7</td>
<td>0.06</td>
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<tr>
<td>Vmax at 1 Hz, V/s</td>
<td>64.6 ± 11.3, n = 6</td>
<td>88.6 ± 9.2, n = 7</td>
<td>0.13</td>
</tr>
<tr>
<td>APD$_{90}$ at 2 Hz, ms</td>
<td>48.0 ± 9.7, n = 5</td>
<td>83.2 ± 18.0, n = 7</td>
<td>0.12</td>
</tr>
<tr>
<td>APD$_{90}$ at 1 Hz, ms</td>
<td>60.5 ± 12.7, n = 6</td>
<td>84.8 ± 21.6, n = 7</td>
<td>0.36</td>
</tr>
<tr>
<td>APD$_{90}$ at 0.17 Hz, ms</td>
<td>63.6 ± 13.2, n = 5</td>
<td>113.7 ± 24.8, n = 6</td>
<td>0.12</td>
</tr>
<tr>
<td>Slope, ms/s</td>
<td>−2.92 ± 1.27, n = 9</td>
<td>9.08 ± 3.60, n = 11</td>
<td>0.0178</td>
</tr>
<tr>
<td>% Cells with EAD</td>
<td>0%, n = 10</td>
<td>50%, n = 12</td>
<td>0.0152*</td>
</tr>
</tbody>
</table>

RMP indicates resting membrane potential; APA, AP amplitude; Vmax, maximal upstroke velocity; and slope, slope of linear relationship between APD$_{90}$ and pacing period.

Values are mean ± SEM. $P$ determined by Student $t$ test except *, which was analyzed by Fisher exact test.

**Discussion**

The aim of the present study was to investigate whether reprogramming of fibroblasts harvested from a representative LQTS 3 model enabled reproduction of the characteristic electrophysiological features of the disease in vitro. For this purpose, we used a well-established LQTS 3 mouse model carrying the human ΔKPQ mutation of the Na⁺ channel (Scn5aΔ+) that is known to display typical electrophysiological features of LQTS 3, including specific frequency-dependent changes of the AP duration.$^{3,12}$

We demonstrated that iPS cells can be generated from wild-type and Scn5aΔ/+ MEFS. The pluripotency of iPS clones was confirmed by gene expression analysis, teratoma formation, and in vitro differentiation assays. Most importantly, wild-type and Scn5aΔ/+ iPS cells could be differentiated into functional intact cardiomyocytes. We investigated different stages of iPS cell differentiation and found that compared with cells in the early developmental stage, late developmental stage cells have higher Scn5α gene expression, and a larger percentage of late developmental stage cardiomyocytes expressed functional Na⁺ currents, which is similar to ES cell–derived cardiomyocytes.$^{11}$ Therefore, late developmental stage spontaneously beating single cardiomyocytes were analyzed, and the biophysical properties between wild-type and Scn5aΔ/+ cardiomyocytes were compared. Cell capacitance, Scn5α channel distribution, and Na⁺ peak current densities were similar between the 2 genotypes; however, in Scn5αΔ/+ cardiomyocytes, Na⁺ currents had faster recovery from inactivation and larger amplitudes of the late component of the Na⁺ current. These biophysical properties are typical of the ΔKPQ mutation and have been described previously in heterologous expression systems$^{4}$ and in cardiomyocytes from the LQTS 3 mouse model.$^{5}$ Other biophysical features of the Na⁺ current, such as steady state activation and inactivation, were unaltered, which is in accordance with previous reports on the ΔKPQ mutation.$^{3,4}$ When measuring APs, we observed a tendency toward longer APD$_{90}$ in Scn5αΔ/+ cardiomyocytes, but this did not reach statistical significance because of high intercellular variability. This variability is most likely because in contrast to investigations of the adult heart in previous studies,$^{3}$ the developmental stages of iPS and ES cell–derived cardiomyocytes is not identical between individual cells. We therefore determined the APD$_{90}$ at different pacing rates in the same cell and found that all Scn5αΔ/+ iPS-derived cardiomyocytes had prolonged APD$_{90}$ at lower stimulation rates and a high incidence of EADs. Thus, cardiomyocytes with the ΔKPQ mutation were characterized by a steep APD$_{90}$–pacing period ratio, and this clearly differed from iPS cell–derived wild-type cardiomyocytes. The steep positive frequency dependence of AP or QT duration is, in contrast to LQTS 1 and 2, a phenotypic hallmark of LQTS 3 and has been reported in mice$^{13}$ and patients.$^{12}$

We were able to analyze APD$_{90}$ in LQTS3–specific iPS cell–derived cardiomyocytes within a broad range (0.5 to 6 seconds) of pacing periods. Although these were substantially lower rates than observed in adult mice, the large pacing range enabled investigations of the frequency-dependent adverse effects of ion channel mutations and of novel compounds, in particular with the use-dependent block of ion channels.

The generation of disease-specific cardiomyocytes from iPS cells represents an important step, because human cardiomyocytes cannot be harvested and expanded in sufficient numbers from patients. Because of this limitation, previous investigations of LQTS used heterologous expression in noncardiomyocytes to unravel the consequences of a mutation on the biophysical properties of the affected ion channel. However, because these cells lack the potential for AP
generation, such systems can only predict the possible consequences related to AP shape and duration in cardiomyocytes. In addition, heterologous systems do not reflect the influence of potential compensatory pathways, the impact of known and unknown accessory channel subunits, and the variability of cell-specific trafficking aspects, all effects that may be present in functional cardiomyocytes. Although mouse models displaying human LQTS have been generated with success and recapitulate at least in part the phenotype in cardiomyocytes, they are of limited relevance because of striking differences in heart rate and because they cannot be used to generate unlimited amount of cardiomyocytes for high-throughput screenings in vitro.

Recently, LQTS 1 and 2 disease–specific cardiomyocytes were obtained from reprogrammed fibroblasts of patients. Here, we provide evidence that LQTS 3 disease–specific iPS cells can also be generated and differentiated into cardiomyocytes that maintain the functional hallmarks of the mutation in vitro. This outcome could not be predicted by previous studies, because iPS-derived cardiomyocytes are not terminally differentiated. At the early developmental stage, ES cell–derived cardiomyocytes have primarily Ca$^{2+}$-driven APs, and only at later stages of differentiation does the functional Na$^{+}$ current density increase and the AP become Na$^{+}$ dependent. In addition, beating frequencies of iPS- and ES-derived cardiomyocytes are similar to fetal hearts, which are characterized by much lower beating rates than adult cardiomyocytes. It was therefore unclear whether the functional phenotype of the KPQ mutation could be reproduced despite these obvious physiological differences between adult mouse and iPS cardiomyocytes. The present data clearly demonstrate that the pathognomonic functional features of the KPQ mutation, namely, the long AP duration at slower pacing rates and the occurrence of EADs, are identified in LQTS 3–specific iPS-derived cardiomyocytes. In future

Figure 4. AP characteristics of LQTS 3–specific and wild-type cardiomyocytes. A and B, Representative traces of APs from wild-type (A) and Scn5a$\Delta/+$(B) cardiomyocytes at high and low pacing frequencies. C, Statistical analysis of APD<sub>90</sub> at different pacing periods for wild-type (WT) and Scn5a$\Delta/+$ cells. D, Relationship between APD<sub>90</sub> and pacing period of the wild-type (black) and Scn5a$\Delta/+$(red) cell shown in panels A and B. Linear regression shown by dashed lines. E, Statistical analysis of the slope of APD<sub>90</sub>-pacing period relationship from individual cells. F, Typical EADs were observed at low pacing frequencies only in Scn5a$\Delta/+$ cells (middle and right, arrows) and never in wild-type cells (left). G, Percentage of cardiomyocytes displaying EADs. Dotted line indicates 0 mV; error bars, SEM. *P < 0.05.
studies, it should be possible to measure these effects with a high-throughput patch clamp or extracellular field potential recordings. Such approaches could enable pharmacological screening in vitro on cardiomyocytes, sparing animal experiments. Moreover, it is very likely that the use of iPS cells will allow functional analysis and patient-specific drug development even in patients in whom the specific mutation underlying the disease has not yet been identified. The feasibility of generating iPS cells from skin biopsy samples, keratinocytes, or somatic cell types of humans will enable the establishment of a wide range of relevant human disease models in the culture dish.

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Disclosures
None.

References

Novelty and Significance
What Is Known?

• The pathophysiological consequences of ion channel mutations that cause long-QT syndrome (LQTS) cannot be analyzed directly in cardiomyocytes from patients.
• Induced pluripotent stem (iPS) cells can be generated from skin biopsy samples of patients and differentiated into cardiomyocytes.

What New Information Does This Article Contribute?

• Disease-specific iPS cells can be generated from murine fibroblasts that carry a human mutation of the Na+ channel that causes LOTS 3.
• Cardiomyocytes can be differentiated in the culture dish from LOTS 3–specific iPS cells and show the known biophysical features of the cardiac Na+ channel mutation.
• Action potential durations of LOTS 3 cardiomyocytes were found to be prolonged at slow heart rates, which is the pathognomonic feature of LOTS 3.

LQTS are characterized by severe, potentially life-threatening cardiac arrhythmias and are caused by ion channel mutations that lead to prolongation of the cardiac action potential duration. The pathophysiological features of LOTS have been investigated by identifying the underlying mutation, analyzing mutated ion channels in nonexcitable cells, and predicting the functional consequences on action potential duration in cardiomyocytes. A much more promising approach would be the direct analysis of cardiomyocytes from LOTS patients. Because it is technically not feasible to harvest sufficient numbers of cells from cardiac biopsy samples, we suggest an alternative approach using cardiomyocytes differentiated from iPS cells that can be obtained from skin biopsy samples by reprogramming. We demonstrate as a proof-of-principle that iPS cell–derived cardiomyocytes can be harvested from a mouse model with a human Na+ channel mutation that causes LOTS 3. Most importantly, for the first time, we analyzed action potentials of LOTS 3–specific cardiomyocytes from iPS cells and identified prolonged action potential durations and early afterdepolarizations at low pacing rates, which is the typical feature of LOTS 3. We suggest that cardiomyocytes differentiated from disease-specific iPS cells are a powerful tool for the investigation of disease mechanisms in vitro and for patient-specific drug screenings.
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SUPPLEMENTAL MATERIAL

Malan et al.: Cardiomyocytes from LQTS 3-specific iPS cells

Detailed Methods:

Generation of iPS cells

To obtain Scn5aΔ/Δ and wild-type mouse embryonic feeder cells (MEFs) Scn5aΔ/+ and wild-type littermates were mated and embryos harvested at embryonic day 13.5. MEFs were generated from individual embryos by Trypsin-digestion as reported earlier.1 MEFs were frozen directly after harvesting and the head of each embryo was used for genotyping (see below).

For iPS cell generation, MEFs with the desired genotype were thawed and cultured in MEF medium (DMEM, 15% FCS, 0.1 mmol/L MEM nonessential amino acids, 0.1 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 mg/mL streptomycin, all from Invitrogen) for less than three passages and seeded at a density of 5 x 10⁴ cells per well of a 6-well plate one day before transduction. Reprogramming was initiated by transduction with ecotropic retroviruses either expressing the three factors Oct4, Sox-2, and Klf4, or additionally c-Myc, as previously described.1 Therefore MEFs were incubated with virus-containing supernatants (250-500 µl of each virus) supplemented with 6 µg/mL protamine sulphate (Sigma) for 24 h (day 0 of reprogramming). The next day, the medium was replaced with fresh MEF medium. At day 4 cells were passaged once and from this time on further maintained in mouse ES cell medium (MEF medium supplemented with 1000 U/mL leukemia inhibitory factor (Chemicon), 3 µmol/L CHIR99021 and 1 µmol/L PD184352 (Axon Medchem)). The iPS cell colonies with ES cell-like morphology were mechanically isolated from day 14 onwards, dissociated with Trypsin, and replated on irradiated MEF layers in mouse ES cell medium. Genotyping of Scn5aΔ/+ embryos, MEFs and iPS cells was performed as reported earlier.2

For retrovirus production pMXs-based retroviral vectors1 (kindly provided by S. Yamanaka through Addgene) encoding the mouse cDNA of Oct4, Sox-2, Klf4 and c-Myc was used. Each retrovirus was produced individually. Therefore HEK 293 FT cells (Invitrogen) grown to ~70% confluence on a 10 cm dish were co-transfected with a pMXs vector (2 µg) and a packaging-defective ecotropic helper plasmid (2 µg, pCL-Eco, kindly provided by I.M. Verma through Addgene) using Fugene 6 transfection reagent (12 µl, Roche) according to manufacturer’s recommendations. Virus supernatants (10 ml each 10 cm plate) were harvested 48 h after transfection and passed through a 0.45-µm filter. Retrovirus production and infection was controlled using a pMXs-GFP control virus in parallel.
Culture and differentiation of iPS cells
The iPS cells were cultured in mouse ES cell medium (see above) on irradiated MEF layers and passed every 2 to 3 days. For cardiomyocyte differentiation the hanging drop method was performed to initiate embryoid body (EB) formation (day 0 of differentiation) as previously described. Briefly, EBs were generated by aggregation of 400 cells in 20 µL of differentiation medium for 2 days and subsequent cultured for another 5 days in differentiation medium (Iscove’s modified Dulbecco’s medium, 20% FCS, 0.1 mmol/L MEM nonessential amino acids, 0.1 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin, all Invitrogen). At day 5 or day 7, EBs were plated on glass cover slips or 6-well plates and used for immunocytochemistry at day 10 or dissociated into single cardiomyocytes at early (day 12) and late (day 19-22) developmental stages for patch clamp analysis. Most of the WT and Scn5a∆/+ iPS clones had comparable cardiac differentiation capabilities and clones #7 (WT) and #6 (Scn5a∆/+ ) were used for differentiation experiments in this study.

Genotyping of MEFs and iPS cells
To examine the genotype of the Scn5a∆/+ MEFs and iPS cells, the Scn5a locus was amplified from genomic DNA using forward (5´-GCAGTGGGAGGACAACCTCTACATG-3´) and reverse (5´-GTTCCTCGTGTGGTGAAGTAATAGTGG-3´) primers as previously described. PCR products were subjected to EcoRV digestion which cuts a silent mutation in the mutated Scn5a allele and results in 1.7 kb and 0.7 kb fragments in contrast to the 2.4 kb fragment of the wild-type allele.

Teratoma assay
Teratoma assays were performed for each genotype. Undifferentiated murine iPS cells were obtained by trypsin dissociation. Preplating was performed on 0.1% gelatin-coated dishes for 45 min to obtain a pure iPS cell suspension without feeder cells. We injected 100 µl cell suspensions with 1x10⁶ iPS cells subcutaneously into the hind limbs of SCID mice. Four weeks after the injection, tumors were surgically dissected, fixed over night in Z-Fix (Anatech LTD), and embedded in paraffin. 6 µm sections were cut and stained with haematoxylin and eosin.

Immunocytochemistry
Immunostainings were performed on colonies of undifferentiated iPS cells and EBs at day 10 of differentiation after fixation with 4% paraformaldehyde (Fluka) for 30 min. Cells and EBs were permeabilized with 0.2 % Triton X-100 (Fluka) for 10 min, blocked with 5% normal donkey/goat
serum (Jackson ImmunoResearch) for 30 min, incubated with primary antibodies diluted in 0.5% normal donkey/goat serum for 2 h and subsequently incubated with secondary antibodies diluted in Hoechst (1 µg/mL; Sigma) for 1 h. Samples were embedded in Polyvinyl alcohol mounting medium (Fluka) and analyzed using an AxioObserver Z1 microscope equipped with an ApoTome optical sectioning device and the AxioVision software (Zeiss). Colonies of iPSC cells were stained against Oct4 (rabbit polyclonal IgG, 1:100; Santa Cruz Biotechnologies) and SSEA1 (mouse, 1:80; Developmental Studies Hybridoma Bank). Differentiated EBs were stained against βIII-Tubulin (mouse, 1:100; Sigma-Aldrich), Nestin (mouse, 1:100; Chemicon), Troma1 (rat, 1:100, R. Kemler, Freiburg), platelet endothelial cell adhesion molecule-1 (PECAM-1, rat, 1:800; Becton Dickinson), α-Actinin (mouse, 1:400; Sigma) and anti Nav1.5 (rabbit, 1:200; Alomone). Secondary antibodies used in this study were: donkey anti-mouse IgG Cy2-labeled, donkey anti-mouse IgG Cy3-labeled, donkey anti-rat Cy3-labeled, donkey anti-rabbit Cy3-labeled (all 1:400; all from Jackson ImmunoResearch) and goat anti-mouse IgG Alexa647-labeled (1:500; Invitrogen).

**Quantitative gene expression analysis**

Gene expression was quantified by real time PCR. Total RNA was extracted using the RNeasy plus mini kit (Qiagen) from undifferentiated iPSC cells and differentiated EBs at day 9 and day 14 and 300 - 800 ng RNA was reverse transcribed into cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative polymerase chain reaction was performed in triplicates using Taqman Assays and Gene Expression Master Mix (Applied Biosystems) according to manufacturer's instructions on a Rotorgene 6000 real time polymerase chain reaction machine (Corbett). The following assays were used: Oct4: Mm00658129_gH, Nanog: Mm02019550_s1, Myh6: Mm00440354_m1, Scn5a: Mm00451971_m1, Hcn4: Mm01176086_m1, Kcnb1: Mm00492791_m1, Cacna1c: Mm00437917_m1, Actn2: Mm00473657_m1, Gapdh: Mn99999915_g1. Raw data were analysed with the Rotor-Gene Q Series Software 1.7. Cycle threshold (C\text{T}) was calculated during the exponential phase at identical threshold values for all runs. C\text{T} values of target genes were subtracted by the C\text{T} value of the housekeeping gene Gapdh to obtain ΔC\text{T} and relative expression was calculated as 2ΔC\text{T}. Each analysis was performed with three independent biological replicates from independent differentiations. Relative expression values were normalized to the mean of wild-type undifferentiated iPSC cells (for Oct4 and Nanog) or to the mean of wild-type differentiated EBs at day 14 (for all other genes).
Single cardiomyocyte isolation and electrophysiology

For patch-clamp experiments single beating cardiomyocytes from EBs were used at early (day 12) and late (day 19-22) developmental stages of differentiation. Therefore beating areas of 20-40 EBs were dissected under a microscope, collected in PBS and dissociated with 1mg/mL collagenase type B (Roche) for 25 min at 37°C under shaking conditions. Single cells were plated at low densities on gelatine-coated (0.1%) coverslips in differentiation medium in order to obtain isolated single cells. Patch-clamp experiments were performed after 48-72 h on single beating cardiomyocytes using an EPC10 amplifier (Heka) in the whole cell configuration as reported earlier with continuously superfusion with extracellular solution at 37°C.

Na+ current was measured in the voltage clamp mode. For recording of peak and recovery from inactivation Na+ current (Figure 3 B-E) the pipette solution contained (in mmol/L) 3 NaCl, 133 CsCl2, 2 MgCl2, 2 NaATP, 2 TEACl, 10 EGTA and 5 HEPES (pH7.3 CsOH) and the external solution: 7 NaCl, 133 CsCl2, 1.8 CaCl2, 1.2 MgCl2, 5 Hepes, 11 glucose, 0.005 nifedipine, pH 7.4 (CsOH). Liquid junction potential of these solutions was 0.2 mV and therefore neglected. For the recovery of inactivation kinetics the peaks Na+ current in response to pairs of depolarization pulses from -100 mV to 10 mV were recorded with increasing delays between the two pulses from 1.5 ms to 57 ms). For quantification the Na+ current amplitude from the second pulse was normalized to those from the first pulse, plotted against the delay and these values were fitted with a mono-exponential growth (examples see Figure 3 D). The current densities for the peak Na+ current were measured from the first pulse of the recovery from inactivation protocol, in response to a -10 mV depolarizing pulse of 40 ms from a holding potential of -100 mV. Steady state fast activation was measured by application of 30 ms pluses of increasing amplitude (10mV steps) from -90 mV to 0 mV from a holding potential of -100 mV. Peak currents were divided by the respective driving force for Na+ to obtain the Na+ conductance \( g_{Na} = I_{Na} / \left[ V_{m} - V_{eq} \right] \). Data from of each individual cell was normalized to maximum conductance and fitted using a Boltzmann distribution to obtain the half maximal voltage of activation. To calculate the time constants of Na+ current deactivation, currents at -40 mV and -10 mV were fitted with single exponential function. Steady state inactivation was measured by a double pulse protocol from a holding potential of -100 mV consisting of a 200 ms pulse of increasing amplitude from -120 mV to 0 mV (10 mV steps) followed by a test pulse of 25ms to 0 mV. Na+ currents were normalized to the maximum current and each individual cell was fitted with a Boltzmann distribution to obtain the half maximal voltage of inactivation.

Late Na+ current was measured using an external solution containing (in mmol/L): 135 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 10 Hepes, 10 glucose, 0.005 nifedipine, pH 7.4 (NaOH). The late
tetrodotoxin-sensitive Na\(^+\) current density was measured at the end of a 100ms test pulse to -10mV and 20 sweeps before and after tetrodotoxin (20µM) application were averaged and subtracted. Currents were normalized to the cell capacitance and expressed in pA/pF. Recording of membrane potential was performed in the current clamp mode and with a pipette solution containing (in mmol/L) 50 KCl, 80 K-Asparatate, 1 MgCl\(_2\), 3 MgATP, 10 EGTA, 10 HEPES, pH 7.4 (KOH) and an external solution containing 140 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 10 Hepes, 10 glucose, pH 7.4 (NaOH). Spontaneous membrane potential without current injection was not significantly different between wild-type (-72.2±2.7 mV, n=13) and Scn5aΔ/+ (-69.5±5.6 mV, n=15) cardiomyocytes. In cells with resting membrane potential >-70 mV and/or spontaneous action potential (AP) generation, current injection was used to obtain a resting membrane potential of ~ -80 mV. The required current to achieve this and the resulting resting membrane potential (Table 2) was not significantly different between wild-type (14.2±7.1 pA, n=13) and Scn5aΔ/+ (23.2±8.3 pA, n=15) cardiomyocytes. APs were elicited by a 2.5 ms current injection pulse though the patch pipette and the strength of the pulse was increased stepwise until a stable action potential with an explicit peak was established. The stimulation frequency was controlled by an external Stimulator (Model 2100, A-M Systems) attached to the EPC10 amplifier. To quantify the frequency-dependent AP duration, cardiomyocytes were stimulated at pacing periods between 0.5s and 6s and at each period the average APD at 90% of repolarization was determined. For each individual cell the APD90 values were plotted against the periods and a linear regression analysis was used to determine the slope of this relationship (examples see Figure 4C).

Data were acquired at a sampling rate of 10-20 kHz (voltage clamp) or 5 kHz (current clamp), filtered at 1 KHz, digitized with the Patchmaster software (HEKA) and analyzed offline using the Fitmaster (HEKA) or the Labchart software (AD Instruments). Membrane potentials were not corrected for liquid junction potentials. AP parameters were analyzed with the cardiac action potential analysis module of Labchart. The maximal AP upstroke velocity was determined in the region from 30% to 100% of action potential amplitude in order to minimize the current injection effects in the initial phase of the AP. APD90 was calculated from the peak of the AP to the point where the AP has dropped by 90% of its amplitude.

Statistical tests were performed using unpaired Student’s t-Test for all data expect percentage of cells with early afterdepolarizations which was analyzed by Fisher’s exact test. A \( P \)-value of <0.05 was considered significant and is indicated by a * in the figures. All data is expressed as mean±SEM.
Legends for Movie files

**Supplementary Movie 1.** Movie of a representative embryoid body derived from wild-type iPS cells with a spontaneous beating area. Recording and playback speed is 15 fps.

**Supplementary Movie 2.** Movie of a representative embryoid body derived from Scn5aΔ/+ with a spontaneous beating area. Recording and playback speed is 15 fps.

References

