Inherited arrhythmia syndromes
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Chapter 4

Switch from fetal to adult SCN5A isoform in human induced pluripotent stem cell-derived cardiomyocytes unmasks the cellular phenotype of a conduction disease-causing mutation


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ABSTRACT

Rationale
Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can recapitulate features of ion channel mutations causing inherited rhythm disease. However, the lack of maturity of these cells is considered a significant limitation of the model. Prolonged culture of hiPSC-CMs promotes maturation of these cells.

Objective
We studied the electrophysiological effects of the I230T mutation in the sodium channel gene SCN5A in hiPSC-CMs generated from a homozygous (I230T\textsuperscript{homo}) and a heterozygous (I230T\textsuperscript{het}) individual from a family with recessive cardiac conduction disease. Since the I230T mutation occurs in the developmentally regulated ‘adult’ isoform of SCN5A, we investigated the relationship between the expression fraction of ‘adult’ SCN5A isoform and the electrophysiological phenotype at different time points in culture.

Methods and results
After a culture period of 20 days, \(I_{Na}\) was mildly reduced in I230T\textsuperscript{homo}-hiPSC-CMs compared to control hiPSC-CMs, while I230T\textsuperscript{het}-hiPSC-CMs displayed no reduction in \(I_{Na}\). This coincided with a relatively high expression fraction of the ‘fetal’ SCN5A isoform compared to the ‘adult’ isoform as measured by qPCR. Following prolonged culture to 66 days, the fraction of ‘adult’ SCN5A isoform increased; this was paralleled by a marked decrease in \(I_{Na}\) in I230T\textsuperscript{homo}-hiPSC-CMs, in line with the severe clinical phenotype in homozygous patients. At this time in culture, I230T\textsuperscript{het}-hiPSC-CMs displayed an intermediate loss of \(I_{Na}\) compatible with a gene dosage effect.

Conclusions
Prolonged culture of hiPSC-CMs leads to an increased expression fraction of the ‘adult’ sodium channel isoform. This new aspect of electrophysiological immaturity should be taken into account in studies focussing on the effects of SCN5A mutations in hiPSC-CMs.
INTRODUCTION

The cardiac sodium channel Na\textsubscript{1.5}, encoded by SCN5A, mediates the cardiac sodium current (\textit{I}_{\text{Na}}) and is crucial for the rapid depolarization of cardiomyocytes and impulse propagation in the heart\textsuperscript{1}. Mutations in SCN5A have been associated with a broad spectrum of inherited cardiac rhythm disorders\textsuperscript{2} and electrophysiological studies on these mutations in heterologous expression systems such as human embryonic kidney (HEK) cells have provided insights into the underlying mechanisms. Yet, limitations inherent to these cellular models, such as their non-cardiomyocyte nature and the fact that the mutant channel is over-expressed, have hindered the faithful recapitulation of sodium channel defects underlying these disorders. The ability to derive human cardiomyocytes from induced pluripotent stem cells (hiPSC-CMs)\textsuperscript{3, 4} from patients with these disorders now allows us to study the consequences of SCN5A mutations in the cardiomyocyte setting, promising a refined understanding of the associated mechanisms and faithful models for the discovery of new therapies. Notwithstanding, while a number of studies have demonstrated that hiPSC-CMs can recapitulate the predicted cellular electrophysiological phenotype caused by SCN5A mutations\textsuperscript{5, 6}, it is widely recognized that hiPSC-CMs are relatively immature\textsuperscript{7}. This aspect needs to be considered in the interpretation of data obtained from these cells.

We here revisited the pathophysiology of the I230T (c.689T>C) mutation in SCN5A by studying patient-derived hiPSC-CMs\textsuperscript{8}. In contrast to the vast majority of mutations in SCN5A that display an autosomal dominant inheritance pattern, the I230T mutation displayed recessive inheritance with homozygous carriers being severely affected by sinus node dysfunction (SND), conduction disease, and severe ventricular arrhythmias at young age, whereas heterozygous carriers displayed mild or no symptoms\textsuperscript{8}. Functional data on this mutation in HEK cells overexpressing the mutant channel revealed decreased \textit{I}_{\text{Na}} and shifts in voltage dependence of activation and inactivation. In an effort to refine our understanding of the defects associated with this mutation, we here compared sodium channel function in hiPSC-CMs from a heterozygous and a homozygous carrier of the I230T mutation and two unrelated control individuals. hiPSC-CMs from the homozygous and heterozygous carriers displayed a drastic and a moderate reduction in \textit{I}_{\text{Na}}, respectively, attesting to a mutant allele dosage effect. Interestingly, we observed that the severity of \textit{I}_{\text{Na}} loss and the associated biophysical defects varied with the duration of hiPSC-CM culture, with prolonged culture leading to a more pronounced biophysical defect. Importantly, this effect paralleled the increased expression of the so-called ‘adult’ SCN5A isoform that is most abundant in adult human heart. This ‘adult’ isoform differs from the ‘fetal’ SCN5A isoform in the alternate usage of exon 6. Splicing of exon 6 occurs in a mutually exclusive manner, with inclusion of either the ‘adult’ exon 6 (in which the I230T mutation is present) or the ‘fetal’ exon 6a\textsuperscript{9, 10}. The relatively high
expression of ‘fetal’ SCN5A isoform is a new feature of immaturity that fits with the ‘fetal’ phenotype of hiPSC-CMs. Our study underscores the importance of taking into account this aspect in studies aimed at elucidating the genotype-phenotype relationship in hiPSC-CMs.

METHODS

Generation and characterization of hiPSC

Skin punch biopsies were obtained from a heterozygous and a homozygous carrier of the I230T mutation, following written informed consent and approval by the medical ethics committee of the University of Münster. Fibroblasts obtained from these biopsies were reprogrammed following Melton’s protocol\textsuperscript{11}. Retroviruses were produced in HEK293T cells using Fugene 6 transfection with Addgene plasmids 8454 (VSV-G envelope), 8449 (packaging plasmid), 17217 (OCT4), 17218 (SOX2), 17219 (KLF4), and 17220 (MYC)\textsuperscript{12}. Cell lines displaying typical human embryonic stem cell (hESC) morphology were further characterized according to standard assays\textsuperscript{13}. In brief, mutations were confirmed using Sanger DNA sequencing. Transgene silencing in clonal hiPSC lines was monitored using primers given in Table S1. Karyotypes were determined based on chromosome counting using standard procedures. hESC marker gene expression was assessed using standard RT-qPCR analysis (Table S1). Pluripotency was assessed by spontaneous in-vitro differentiation as embryoid bodies, followed by cell aggregate plating and maturation in serum-containing media. Test differentiation along the cardiac lineage was carried out as described\textsuperscript{14}. Immunocytochemistry was performed according to standard procedures using antibodies α-SMA (Dako #M0851, 1:100), α-AFP (Dako #A0008, 1:300), α-βIII-Tubulin (Sigma #T8660, 1:1000), and α-Actinin (Sigma #A7811, 1:800). One heterozygous and one homozygous hiPSC line showing near-complete transgene silencing and overall hESC-like characteristics according to these assays were used for further studies. The control hiPSC lines (Ctrl1 and Ctrl2), originating from unrelated individuals, were generated using a lentiviral vector carrying the same transcription factors as the retroviruses used for the patient cell lines. The generation and characterization of these hiPSC lines has been described previously\textsuperscript{15,16}.

Differentiation of hiPSCs into cardiomyocytes and dissociation into single cells

All hiPSC lines were expanded and cultured in feeder-free conditions on Matrigel-coated dishes in the presence of chemically defined medium (E8 Essential Medium, Life Technologies). Differentiation of hiPSC to cardiomyocytes (CMs) was performed over the indicated time periods as previously described\textsuperscript{17}. Briefly, undifferentiated hiPSCs were treated with CHIR99021 (12 μmol/L, Selleckchem) for 24 hours, followed by the treat-
ment with the Wnt-inhibitor IWP4 (5 μmol/L, Stemgent) on days 4 and 5. At the stipulated time points (see supplementary figure 1), enrichment for CMs was achieved by substituting the culture medium to DMEM supplemented with lactic acid (4 mmol/L) in absence of glucose for 4–6 days as described previously. For electrophysiological measurements, hiPSC-CMs were enzymatically dissociated into single cells using Elastase (Serva) and Liberase (Roche Chemicals), plated at a low density on Matrigel-coated coverslips and measured 8–11 days after dissociation. For gene expression analysis, cells were lysed directly upon the 4–6 days of lactate treatment. Samples for electrophysiological measurements and RNA expression analysis were matched for their duration in culture (supplementary figure 1).

RNA isolation and SCN5A isoform expression assay
RNA was isolated with the Nucleospin RNA isolation kit according to the manufacturer’s protocol from at least 3 independent hiPSC-CM differentiations of each of the three hiPSC lines using the Nucleospin RNA isolation kit (Machery-Nagel) (supplemental figure 1). The adult heart RNA was obtained from 3 samples of non-diseased ventricular tissue obtained from an adult donor whose hearts were explanted for heart transplantation but were not used due to logistical reasons. For these samples, Trizol (Invitrogen) was used according to the manufacturer’s instructions. cDNA was generated from 500 ng of RNA by reverse transcriptase (Superscript II, Life Technologies) using oligoDT primers. To determine transcript abundance, quantitative PCR (qPCR) was conducted with SYBR green on a Roche LightCycler 480 Real-Time PCR System. All measurements were conducted in triplicate. Gene expression levels were analyzed using the LinReg PCR program. To distinguish transcripts corresponding to the ‘fetal’ and ‘adult’ SCN5A isoforms, we used isoform-specific forward primers that selectively anneal to the fetal or adult exon 6 together with a reverse primer in exon 7 (see Figure 3A). For calibration purposes, we included in the same plate samples with predetermined template ratios of 1:8, 1:4, 1:2, 1:1, 2:1, 4:1 and 8:1 of adult:fetal SCN5A. For this purpose we first cloned the PCR products corresponding to the specific isoforms into the PCR2.1 TOPO vector and subsequently quantified the concentration of the generated plasmid using the QuantiT PicoGreen dsDNA assay kit (Thermo Fisher) in three independent experiments. The primers, which were intron-spanning, are depicted in Table S1. CT values above 35 were considered to be indicative of transcripts below the detection limit. Specificity of the primers for all amplicons was confirmed by Sanger sequencing of the amplicons.

Minigene assay
For the minigene assay, a genomic region from nucleotide 38,654,626 to nucleotide 38,656,642 of chromosome 3 (numbering according to human genome assembly hg19; extending from 1091 bp upstream of exon 6a to 614 bp downstream of exon 6,
see Figure 4A) was amplified using genomic DNA from the hiPSC Ctrl1 line as template and subsequently cloned into the plasmid RHCglo (kindly provided by Dr. T.A. Cooper, Baylor College of Medicine)\textsuperscript{32}. The SCN5A c.689T>C (I230T) mutation was introduced by means of the QuikChange mutagenesis kit according to the manufacturer’s instructions, after which the sequence of the whole plasmid was verified by Sanger sequencing. The construct was transfected into the cardiac cell line H10\textsuperscript{23} using Lipofectamine 2000. RNA was isolated 2 days after transfection by means of the Nucleospin RNA isolation kit (Machery-Nagel). After generating cDNA by reverse transcriptase (Superscript II, Life Technologies) using 500 ng of RNA, exon usage was evaluated by RT-PCR with primers A and D that annealed to the plasmid exons located 5’ and 3’ of exon 6a and 6, respectively, and by qPCR using primer sets A-B and C-D that were exon specific for exons 6a or 6, respectively (Figure 4A).

**Cellular electrophysiology**

*Data acquisition and analysis*

Action potentials (APs) and $I_{Na}$ were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Voltage control, data acquisition, and analysis were realized with custom software. The liquid junction potentials were 15 and 2.5 mV for AP and $I_{Na}$ measurements, respectively, and potentials were corrected accordingly. Signals were low-pass-filtered with a cutoff of 5 kHz and digitized at 40 and 20 kHz for for APs and $I_{Na}$, respectively. Cell membrane capacitance ($C_m$) was determined at a -5 mV voltage step from -40 mV by dividing the time constant of the decay of the capacitive transient by the series resistance. For all cell lines and experiments, data were collected from at least 3 independent differentiations.

*Action potentials*

APs were recorded at 36 ± 0.2°C from single hiPSC-CMs using the amphotericin-B-perforated patch-clamp technique. Spontaneously beating hiPSC-CMs demonstrating regular and synchronous contractions at 3–10 s were selected. Patch pipettes (borosilicate glass; resistance ≈2.0 MΩ) contained (in mmol/L): 125 K-gluconate, 20 KCl, 5 NaCl, 0.44 amphotericin-B, 10 HEPES; pH 7.2 (KOH). Bath solution was composed of (in mmol/L): 140 NaCl, 5.4 KCl, 1.8 CaCl$₂$, 1.0 MgCl$₂$, 5.5 glucose, 5.0 HEPES; pH 7.4 (NaOH). To overcome the lack of the inward rectifier potassium current, $I_{K1}$, which is a typical feature of hiPSC-CMs that limits the functional availability of $I_{Na}$ and transient outward potassium current $I_{To}$\textsuperscript{24,25}, we injected an *in silico* $I_{K1}$ with kinetics of Kir$₂.¹$ channels through dynamic clamp, as previously described\textsuperscript{19}. An amount of 2 pA/pF peak outward current was applied, resulting in quiescent hiPSC-CMs with a resting membrane potential (RMP) of -80 mV or more negative. APs were elicited at 1 Hz by 3 ms, ~1.2× threshold current pulses through the patch pipette. AP parameters that were characterized were RMP, maximum
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AP amplitude (APA\textsubscript{max}), AP duration at 20, 50 and 80\% of repolarization (APD\textsubscript{20}, APD\textsubscript{50} and APD\textsubscript{80} respectively), maximal upstroke velocity (V\textsubscript{max}) and plateau amplitude (APA\textsubscript{plateau}, measured 20 ms after the AP upstroke). Averages were taken from 10 consecutive APs.

Sodium currents

$I\textsubscript{Na}$ was recorded in the whole-cell configuration of the patch clamp technique using voltage clamp protocols as depicted in the figures. $I\textsubscript{Na}$ was defined as the difference between peak and steady state current. In all protocols, a holding potential of -100 mV and a cycle length of 5 seconds was used. For $I\textsubscript{Na}$ density and voltage dependence of activation, depolarizing pulses were applied ranging from -100 mV to 30 mV (see the inset of figure 2A). For voltage dependence of inactivation, currents were determined at -20 mV preceded by a conditional step ranging from -120 mV to -20 mV. Recovery of inactivation was measured using a two-step pulse protocol to -20 mV, by which the interpulse interval varied between 1 and 1000 ms. Currents at the second pulse were normalized to currents at the first pulse and plotted against the interval between the two pulses (see the inset of figure 4D). To ensure proper voltage control, $I\textsubscript{Na}$ was measured at room temperature with a low extracellular Na\textsuperscript{+} concentration and series resistances were compensated for ≥ 85\%. Pipette solution contained (in mmol/L): 3.0 NaCl, 133 CsCl, 2.0 MgCl\textsubscript{2}, 2.0 Na\textsubscript{2}ATP, 2.0 TEACl, 10 EGTA, 5.0 HEPES; pH 7.2 (CsOH). Bath solution contained (in mmol/L): 20 NaCl, 120 CsCl, 1.8 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 11.0 glucose, 5.0 HEPES; nifedipine 0.01; pH 7.4 (CsOH). $I\textsubscript{Na}$ density was calculated by dividing current by $C\textsubscript{m}$. Cells that lacked $I\textsubscript{Na}$ completely (in < 5\% of the cells) were excluded from analysis. Steady-state activation and inactivation curves were fitted by using a Boltzmann equation: \( I/I\textsubscript{max} = A/[1.0 + \exp[(V_{1/2} - V)/k]] \), in which $V_{1/2}$ is half-maximum (in)activation potential and $k$ is the slope factor. Recovery from inactivation and the speed of current inactivation were fitted with a bi-exponential function: \( y = y_0 + A_f[1 - \exp[-t/\tau_f]] + A_s[1 - \exp[-t/\tau_s]] \), where $A_f$ and $A_s$ represent the amplitudes of the fast and slow components and $\tau_f$ and $\tau_s$ indicate the time constants of fast and slow components, respectively.

Statistical analysis

Statistical analysis was conducted with SPSS Statistics 22. Normality was tested by visual inspection of histograms of the data and by Shapiro-Wilk tests and equality of variance was assessed by Levene’s test. For normally distributed parameters, groups were compared by two-sided t-tests for two groups or One-Way ANOVA for multiple groups followed by Bonferroni post hoc tests. Mann-Whitney U tests and Kruskal-Wallis tests were applied for non-normally distributed parameters. Data are presented as mean±standard error of the mean (SEM). Statistical significance is defined by \( p < 0.05 \).
Figure 1. Clinical characteristics and characterization of human induced pluripotent stem cells (hiPSCs) generated from the heterozygous I230T mutation carrier (I230T^{het}) and the homozygous I230T mutation carrier (I230T^{homo}). A. Baseline ECG of the homozygous I230T mutation carrier, showing a bradycardia, 1\textsuperscript{st} degree AV-block and marked QRS-prolongation. Scale bar indicates 1 mV (vertical) and 200 ms (horizontal). B. I230T^{het} and I230T^{homo} exhibited normal karyotypes. C. Expression of the transgenes OCT4, SOX2, KLF4 and MYC was silenced in the selected clones. D. Immunostainings of AFP, SMA and β-III tubulin in spontaneously differentiated hiPSCs demonstrating presence of cells from the three germ layers endoderm, mesoderm and ectoderm, respectively. E. Quantification of pluripotency gene expression relative to their expression in the human embryonic stem cell (hESC) line NCL3, demonstrating similar expression levels. F. Immunostaining of α-actinin after directed differentiation of the hiPSCs into cardiomyocytes.

RESULTS

Patient characteristics and generation of hiPSCs

Skin biopsies were obtained from two family members of a previously reported consanguineous family that includes individuals carrying the c.689T>C (I230T) mutation in SCN5A in the heterozygous or homozygous state\textsuperscript{8}. The first donor was a 16 year-old female homozygous mutation carrier, who was first diagnosed at the age of 4 with sick sinus syndrome without any clinical symptoms. The initial Holter ECG showed sinus...
bradycardia (minimal heart rate 35 beats per minute) with atrioventricular block of II-III°, marked QRS prolongation and episodes of tachycardia due to atrial fibrillation with heart rates of 144–202 beats per minute. She was treated with sotalol and received a pacemaker at the age of 5. A follow-up ECG showed normal sinus rhythm and sotalol treatment was stopped. Her baseline ECG is depicted in Figure 1A. The second donor was the 43 year-old mother of the patient described above who is a heterozygous carrier of the same SCN5A mutation. She showed no signs of sinus conduction disease and never displayed symptomatic arrhythmia or syncope in the past. Also, presence of latent Brugada syndrome was excluded by ajmaline challenge. Skin fibroblasts of these two patients were reprogrammed into hiPSCs (annotated as I230Thet and I230Thomo for the heterozygous and homozygous patient, respectively). We confirmed the karyotype and verified that expression of the transgenes MYC, OCT4, SOX2, and KLF4 were silenced in the selected clones (Figure 1B-C). Pluripotency was confirmed by immunostainings in spontaneously differentiated cultures, demonstrating expression of protein markers from the three germ layers, endoderm, ectoderm and mesoderm. Moreover, robust expression of pluripotency genes was confirmed by qPCRs in the hiPSC lines, in which expression levels were similar to the expression levels in the established human embryonic stem cell line NCL3 (Figure 1E). The ability of the hiPSCs to differentiate into cardiomyocytes was confirmed by α-actinin stainings after directed differentiation (Figure 1F). The presence of the c.689T>C mutation was confirmed by Sanger sequencing. For the control hiPSC lines (Ctrl1 and Ctrl2), the generation and complete characterization have been described previously.

Mild electrophysiological defects in I230Thet- and I230Thomo hiPSC-CMs after short-term culture

hiPSC were differentiated into cardiomyocytes for 8 days, treated with lactate for 4–5 days and dissociated into single cells. Electrophysiological parameters were measured 8–11 days after dissociation. Figure 2A shows typical $I_{Na}$ recordings; average current densities, activation, and inactivation characteristics are summarized in Figure 2B-D. In contrast to the severe conduction disease phenotype in the homozygous mutation carrier from which they were derived, I230Thomo hiPSC-CMs displayed only mild $I_{Na}$ and AP abnormalities. At potentials of -55 to -35 mV $I_{Na}$ density was slightly reduced compared to Ctrl1 and Ctrl2 ($p < 0.05$), and maximal peak current density was reduced by approximately 30%, although this difference did not reach statistical significance. (Figure 2B). Voltage dependence of activation of I230Thomo hiPSC-CMs was shifted by 5 mV towards more depolarized potentials, while voltage dependence of inactivation was unchanged (Figure 2C,D). All these $I_{Na}$ parameters did not differ between I230Thet hiPSC-CMs and the two Ctrls (Figure 2B-D). Figure 2E shows typical APs; average AP characteristics are summarized in Figure 2F-H. APs were measured with in silico injection of $I_{K1}$ by dynamic
clamp. Consequently, APs had RMP negative to -80 mV and upstroke velocities of > 100 V/s, indicating an \( I_{Na} \) driven AP upstroke, as reported previously\(^{19, 26} \). Although a trend towards a difference in \( V_{\text{max}} \) was observed (\( p = 0.056 \), ANOVA), none of the AP parameters differed significantly between Ctrl1, Ctrl2, I230T\(^{\text{het}}\) and I230T\(^{\text{homo}}\) hiPSC-CMs (Figure 2F-H). In summary, the mild electrophysiological abnormalities of I230T\(^{\text{homo}}\)-hiPSC-CMs

![Figure 2](image_url)

Figure 2. Initial assessment of sodium currents (\( I_{Na} \)) (A-D) and action potentials (APs) (E-H) in hiPSC-CMs harboring the homozygous and heterozygous SCN5A mutation I230T (I230T\(^{\text{homo}}\) and I230T\(^{\text{het}}\), respectively) as compared to hiPSC-CMs from the two control cell lines (Ctrl1 and Ctrl2). A. Typical traces of \( I_{Na} \) in hiPSC-CMs from Ctrl1 (\( n = 28 \)), Ctrl2 (\( n = 23 \)), I230T\(^{\text{het}}\) (\( n = 31 \)) and I230T\(^{\text{homo}}\) (\( n = 30 \)) as determined with the voltage clamp protocol depicted in the left panel (with cycle length of 5 seconds). B. Average current-voltage relationships of \( I_{Na} \). # and * indicate \( p < 0.05 \) versus Ctrl1 and Ctrl2, respectively (Kruskal-Wallis test followed by Bonferroni corrected pairwise comparisons). C. Voltage dependence of activation demonstrates a depolarizing shift in half maximum voltage of activation (\( V_{1/2} \)) of 5 mV in I230T\(^{\text{homo}}\) compared to the other groups (\( p < 0.05 \); ANOVA, Bonferroni post hoc test) D. Voltage dependence of inactivation (Ctrl1, \( n = 17 \); Ctrl2, \( n = 20 \); I230T\(^{\text{het}}\), \( n = 21 \); I230T\(^{\text{homo}}\), \( n = 15 \)). E. Representative APs measured at 1 Hz in Ctrl1 (\( n = 16 \)), Ctrl2 (\( n = 15 \)), I230T\(^{\text{het}}\) (\( n = 13 \)) and I230T\(^{\text{homo}}\) (\( n = 13 \)). The inset shows the first derivative of the initial phase of the AP. F-H Average action potential characteristics.
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contradicted with the severe conduction disturbances observed in the homozygous patient. They also contrasted with the previously reported electrophysiological data obtained in HEK293 cells overexpressing the mutated channel, in which a severe loss-of-function in $I_{Na}$ (a reduction of 70% in current density and a depolarizing shift of $+15 mV$ in $V_{1/2}$ of activation) was demonstrated. We reasoned that the immaturity of the hiPSC-CMs used in this experiment might have played a role.

The fraction of ‘adult’ to ‘fetal’ SCN5A isoform in hiPSC-CMs increases with prolonged culture

We noted that the SCN5A mutation I230T is located in exon 6, an alternatively spliced exon of the SCN5A pre-mRNA in human heart. The developmentally timed alternative splicing of exon 6 generates two splice isoforms of SCN5A, one containing the canonical exon 6 (the ‘adult’ isoform) and one containing exon 6a (the ‘fetal’ isoform), which differ by seven amino acid residues within the S3 and S4 (voltage sensor) transmembrane segments of the first domain (D1/S3-S4) (Figure 3A)\(^9,^{10}\). As the I230T mutation is located in the canonical exon 6 we hypothesized that the mild electrophysiological phenotype we observed in the mutant hiPSC-CMs was related to this splice event. Since hiPSC-CMs are relatively immature, we posited that the hiPSC-CMs we measured primarily expressed the fetal isoform of SCN5A which does not contain the mutation. Accordingly, we next determined the fractions of ‘fetal’ and ‘adult’ SCN5A isoforms in Ctrl1, Ctrl2, I230T\(^{het}\) and I230T\(^{homo}\) hiPSC-CMs, and compared them to isoform fractions in adult human heart samples (Figure 3B). All groups had significantly lower percentage of ‘adult’ isoform expression compared to adult human heart samples, in which expression of the fetal isoform was virtually absent. The relatively low expression of the ‘adult isoform’ in these hiPSC-CMs could mask the full effects of the I230T mutation, which could underlie the mild electrophysiological phenotype we observed in the studies described above (Figure 2). Interestingly, both I230T\(^{het}\) and I230T\(^{homo}\) exhibited higher fractions of ‘adult’ SCN5A isoform compared to Ctrl1 and Ctrl2, with I230T\(^{homo}\) demonstrating the highest fraction.

Since a number of reports have documented the stimulating effect of extended time in culture in promoting the maturation state of hiPSC-CMs\(^{27-29}\), we next evaluated mRNA fractions of ‘fetal’ and ‘adult’ SCN5A isoforms after prolonged culture. In Ctrl1 hiPSC-CMs we observed a gradual increase in the ‘adult’ SCN5A isoform fraction with extended time in culture, eventually reaching a percentage of ~50% after 66 days (Figure 3C). An increase in adult SCN5A fraction was also observed in Ctrl2, I230T\(^{het}\) and I230T\(^{homo}\) hiPSC-CMs, reaching 57, 76 and 87%, respectively, at day 66 (Figure 3D).
The c.689T>C (I230T) SCN5A mutation enhances usage of ‘adult’ exon 6

We observed increased fractions of ‘adult’ SCN5A isoform expression in the two mutant cell lines, as compared to the two controls (Figure 3B). This difference could either relate to line-to-line variability, or it could relate to the effect of the c.689T>C mutation on alternate usage of exon 6 over exon 6a. In support of the latter, using in silico prediction tools, we noted that the c.689T>C mutation introduces a splice enhancer motif (CTATATC->CTACATC) which could favor the usage of exon 6. We therefore tested this possibility by means of a minigene splice assay. For this, wildtype and mutant minigene constructs were generated encompassing exon 6a, exon 6 and flanking regions (Figure 3).

Figure 3. Expression fractions of adult and fetal SCN5A isoform in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). A. Alternative splicing of exon 6 in SCN5A. Positions of the isoform-specific primers are depicted. B. Average adult and fetal SCN5A fractions in hiPSC-CMs cultured for 20 days from Ctrl1 (n = 6), Ctrl2 (n = 3), I230T<sup>het</sup> (n = 3), I230T<sup>homo</sup> (n = 4) and from adult human heart samples (n = 3). The sum of the total fetal and adult transcripts detected is set to 1. Adult SCN5A fractions of all groups of hiPSC-CMs are lower than the fractions in adult heart, in which expression of the fetal isoform is virtually absent. C. SCN5A isoform fractions in Ctrl1 hiPSC-CMs after 20 days (20d; n = 6), 36 days (36d; n = 6), 51 days (51d; n = 4) and 66 days (66d; n = 7), demonstrating a gradual increase in adult isoform expression. D. Comparison of SCN5A fractions at 20d and 66d in culture in hiPSC-CMs from Ctrl1 (n = 6,7), Ctrl2 (n = 3,3) I230T<sup>het</sup> (n = 3,6) and I230T<sup>homo</sup> (n = 4,4). An increase in adult isoform expression is observed in all groups upon extended time in culture. * indicate p < 0.05 (ANOVA, posthoc Bonferroni tests).
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These were subsequently expressed in the cardiac cell line H10 followed by RT-PCR to assess splicing. We detected RT-RNA fragments of two different sizes for both wildtype and c.689T>C. Sequencing of these two RT-PCR products revealed that one included only the ‘fetal’ exon 6a (190 bp) while the other included both the fetal as well as the adult exon 6 (exon 6a plus exon 6, 270 bp) (Figure 4A, lower panel). Compared to wildtype, c.689T>C samples displayed markedly greater levels of the RT-PCR fragment containing both exons and lower levels of the RT-PCR product containing only exon 6a, supporting a role for the c.689T>C mutation in promoting the inclusion of adult exon 6. This was confirmed by means of exon-specific qPCRs which demonstrated increased expression fractions of the ‘adult’ exon in cells expressing the c.689T>C construct as compared to wildtype (Figure 4B). This supports the notion that the increased ‘adult’ SCN5A isoform fractions that we observed in I230T<sup>het</sup> and I230T<sup>homo</sup> hiPSC-CMs were related to effects of the c.689T>C mutation on splicing.

**Figure 4.** Minigene assay demonstrating increased inclusion of the ‘adult’ exon 6 in constructs carrying the c.689T>C mutation. A. The construct that was overexpressed in H10 cells to evaluate exon usage. The area in grey depicts the genomic fragment from the SCN5A gene that was cloned in the RHC glo vector. RT-PCR on cDNA that was derived from the overexpressed samples using primers A and D at the depicted areas yielded two products (lower panel), which consisted of either inclusion of only exon 6a or inclusion of both exons 6a and 6. B. Expression fractions of exon 6 and exon 6a in samples overexpressed with wild-type (WT) and c.689T>C mutant (n = 3–4, two independent experiments), as determined with exon specific quantitative PCRs using primer sets A-C for exon 6a and primer sets B-D for exon 6. The c.689T>C mutation significantly increases usage of exon 6 (p < 0.05, t-test). Action potential duration (F), maximal upstroke velocity (Vmax; G), resting membrane potential (RMP) and action potential amplitude (APA) (H) were not statistically different between all groups.

**Changes in I<sub>Na</sub> properties upon extended time in culture**

The adult isoform of the SCN5A-encoded channel Na<sub>v</sub>1.5 exhibits different biophysical properties compared to the fetal one. In heterologous expression systems, a hyperpolarizing shift in V<sub>1/2</sub> of activation, and faster rates of current inactivation are consistently found in channels translated from the adult SCN5A isoform compared to the fetal one, while some report a decreased current density and a faster recovery rate from inac-
tivation as well\textsuperscript{10, 31, 32}. To evaluate whether these changes in $I_{\text{Na}}$ parameters occur in hiPSC-CMs expressing increased fractions of adult $SCN5A$, we determined properties of $I_{\text{Na}}$ in both Ctrl1 and Ctrl2 hiPSC-CMs at 'late'-stage (66–69 days) and compared them to those measured in the same lines at ‘early’-stage (20–23 days). All parameters are summarized in Table 1 and Supplementary Figures 2 and 3. A marked increase in $I_{\text{Na}}$ density was observed in both control lines upon extended time in culture ($p < 0.01$), while voltage dependence of activation remained unchanged. In Ctrl2 hiPSC-CMs, voltage dependence of inactivation displayed a positive shift of +3 mV upon extended time.

Figure 5. Extended time in culture unmasks the electrophysiological phenotype in I230T\textsuperscript{het} and I230T\textsuperscript{homo} hiPSC-CMs. A. Representative $I_{\text{Na}}$ traces in hiPSC-CMs from Ctrl1 (n = 31), Ctrl2 (n = 28) I230T\textsuperscript{het} (n = 26) and I230T\textsuperscript{homo} (n = 22). B. Average current-voltage relations of $I_{\text{Na}}$. * indicate $p < 0.01$ versus Ctrl1, Ctrl2 and I230T\textsuperscript{het} (Kruskal-Wallis test followed by Bonferroni corrected pairwise comparisons). C, D. Voltage dependence of activation and inactivation, respectively. E. Typical examples of APs measured at 1 Hz (Ctrl1, n = 16; Ctrl2, n = 19; I230T\textsuperscript{het}, n = 13; I230T\textsuperscript{homo}, n = 13). F-H Average AP characteristics, including action potential duration (F), maximal upstroke velocity ($V_{\text{max}}$; G) resting membrane potential (RMP) and action potential amplitude (APA) (H).
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in culture, but remained unchanged in Ctrl1 hiPSC-CMs. Also, the slow component of recovery from inactivation was faster in ‘late’ stage hiPSC-CMs compared to ‘early’ stage hiPSC-CMs in Ctrl1 (p = 0.037), while in Ctrl2 the recovery from inactivation was similar at both stages. In both groups, inactivation rate of $I_{Na}$, measured at -30 mV was increased in ‘late’-stage versus ‘early’-stage hiPSC-CMs (p < 0.001 in both groups).

**Prolongation of the time in culture unmasksthe phenotype of heterozygous I230T and homozygous I230T hiPSC-CMs**

Having found an increased fraction of adult SCN5A isoform expression in hiPSC-CMs upon extended time in culture and considering the fact that the I230T mutation is located in the canonical exon 6 which is incorporated into the adult isoform, we expected a more pronounced electrophysiological phenotype in the mutant cell lines after culturing for a time period of 66 days. Indeed, after prolonged culture $h_{Na}$ density was markedly reduced by 85% in I230T homo hiPSC-CMs compared to Ctrl1 and Ctrl2 hiPSC-CMs which were cultured for a similar period of time (p < 0.01), with I230T het hiPSC-CMs displaying an intermediate reduction (Figure 5A, B). Moreover, $V_{1/2}$ of activation was shifted in the positive direction by +10–11 mV in I230T homo-hiPSC-CMs compared to Ctrl1 and Ctrl2 (p < 0.05), while I230T het- hiPSC-CMs exhibited a significant shift in $V_{1/2}$ of activation of +2.4 mV compared to Ctrl1 (p < 0.05). (Figure 5C). Finally, voltage dependence of inacti-

### Table 1. Sodium current ($I_{Na}$) properties in ‘early’ and ‘late’-stage hiPSC-CMs from the control cell lines.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl1</th>
<th></th>
<th>Ctrl2</th>
<th></th>
</tr>
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<tr>
<td></td>
<td>“Early”-stage</td>
<td>“Late”-stage</td>
<td>“Early”-stage</td>
<td>“Late”-stage</td>
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<tr>
<td>Peak current density (Med±IQR)</td>
<td>-93±78 (n=28)</td>
<td>-207±275* (n=31)</td>
<td>-109±96 (n=23)</td>
<td>-184±164* (n=27)</td>
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<tr>
<td>Voltage dependence of activation</td>
<td></td>
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<tr>
<td>K (mean±SEM)</td>
<td>7.5±0.1 (n=28)</td>
<td>6.9±0.2 (n=31)</td>
<td>6.9±0.2 (n=22)</td>
<td>7.1±0.2 (n=27)</td>
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<tr>
<td>V1/2 (mean±SEM)</td>
<td>-35.0±0.7</td>
<td>-34.4±0.7</td>
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<td>-33.5±0.7</td>
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<tr>
<td>Voltage dependence of inactivation</td>
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<tr>
<td>K (mean±SEM)</td>
<td>-6.9±0.2 (n=17)</td>
<td>-6.3±0.2 (n=14)</td>
<td>-6.9±0.2 (n=20)</td>
<td>-6.6±0.1 (n=23)</td>
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<tr>
<td>V1/2 (mean±SEM)</td>
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<td>-86.0±0.8</td>
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<td>-81.1±1.1*</td>
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<tr>
<td>Tfast (mean±SEM)</td>
<td>54.4±7.7 (n=7)</td>
<td>40.0±10.9 (n=6)</td>
<td>98.0±10.1 (n=13)</td>
<td>83.2±8.6 (n=12)</td>
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<td>Tslow (mean±SEM)</td>
<td>408±19.6</td>
<td>297±37.1*</td>
<td>837±122</td>
<td>773±49</td>
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<tr>
<td>Time rate of inactivation</td>
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<tr>
<td>Tfast (Med±IQR)</td>
<td>1.6±0.6 (n=28)</td>
<td>1.1±0.4* (n=31)</td>
<td>1.6±0.6 (n=23)</td>
<td>1.4±0.6 (n=27)</td>
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<tr>
<td>Tslow (Med±IQR)</td>
<td>5.8±7.25</td>
<td>4.1±5.0*</td>
<td>6.3±7.0</td>
<td>4.3±4.5*</td>
</tr>
</tbody>
</table>

Med, Median; IQR, interquartile range; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes

*p<0.05 Mann-Whitney U test compared to “early-stage” hiPSC-CMs

#p<0.05 Student’s t-test compared to “early-stage” hiPSC-CMs
Table 2. Sodium current ($I_{\text{Na}}$) parameters of all hiPSC lines measured at day 22-25 (early-stage) and at day 66-69 (late-stage). Current density, voltage dependence of activation and are depicted. Data are presented as mean±SEM or median±interquartile range as indicated. Med, median; IQR, interquartile range; κ, slope factor; $V_{1/2}$, half-maximum voltage of (in)activation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Early-stage hiPSC-CMs</th>
<th>Late-stage hiPSC-CMs</th>
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<tr>
<td>Peak current density</td>
<td>Ctrl1</td>
<td>Ctrl2</td>
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<tr>
<td>(Med±IQR)</td>
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<td></td>
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<td></td>
<td>-93±78 (n=28)</td>
<td>-109±96 (n=23)</td>
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<tr>
<td>Voltage dependence of activation</td>
<td>κ (mean±SEM)</td>
<td>7.5±0.1 (n=28)</td>
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<td></td>
<td>-35.0±0.7 (n=17)</td>
<td>-34.8±0.8 (n=20)</td>
</tr>
<tr>
<td>Voltage dependence of inactivation</td>
<td>κ (mean±SEM)</td>
<td>-6.9±0.2 (n=20)</td>
</tr>
<tr>
<td></td>
<td>-85.4±0.9 (n=20)</td>
<td>-84.3±0.9 (n=20)</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to all other groups, Kruskal-Wallis test, followed by Bonferroni corrected pairwise comparisons

# p<0.05 compared to all other groups, One-Way ANOVA followed by posthoc Bonferroni tests

a p<0.05 compared to Ctrl1

b p<0.05 compared to Ctrl1 and I230Thet
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vation in I230T\textsuperscript{homo} hiPSC-CMs was different as compared to I230T\textsuperscript{het} and Ctrl1 (p < 0.05), while Ctrl2 hiPSC-CMs demonstrated similar values as I230T\textsuperscript{homo} hiPSC-CMs (p < 0.05) (Figure 5D). The average $I_{\text{Na}}$ parameters of all lines at the two different stages are summarized in Table 2. AP measurements showed a significant decrease in $V_{\text{max}}$ in I230T\textsuperscript{homo}-hiPSC-CMs compared to Ctrl1, Ctrl2 and I230T\textsuperscript{het} (p < 0.05 I230T\textsuperscript{homo} vs. all groups) in line with the loss-of-function in $I_{\text{Na}}$ (Figure 5E,G). Again, I230T\textsuperscript{het} hiPSC-CMs showed intermediate values (p < 0.05. (Figure 5F).

DISCUSSION

Immaturity of hiPSC-CMs has been widely acknowledged as one of the main limitations of this model in the cardiac research field. Different aspects of immaturity have been described, which include the electrophysiological, structural and the contractile properties of hiPSC-CMs\textsuperscript{7}. With respect to electrophysiology, lack of expression of the inward rectifier potassium current $I_{\text{K1}}$, is considered an important drawback\textsuperscript{33}, but differences in other currents compared to adult cardiomyocytes have also been described\textsuperscript{33, 34}. We here highlight another aspect of electrophysiological immaturity in hiPSC-CMs, stemming from the expression of the fetal splice isoform of the main cardiac sodium channel gene SCN5A that contains the alternatively spliced exon 6a instead of the exon 6 contained in the adult isoform. Moreover, we show that extended culture of hiPSC-CMs leads to a relative increase in the adult isoform over the fetal. We illustrate the relevance of this phenomenon through electrophysiological studies of hiPSC-CMs harboring the SCN5A mutation I230T in exon 6, where we observed mild biophysical defects after short-term culture and uncovered the expected pronounced biophysical defects with extended culture.

The fetal isoform of SCN5A differs from the adult isoform in 7 amino acids within the S3 and S4 transmembrane segment of the first domain\textsuperscript{10}. Previous studies conducted in HEK cells or Xenopus oocytes expressing cDNA encoding either “fetal” or “adult” Na\textsubscript{v}1.5 have consistently reported a hyperpolarizing shift in voltage dependence of activation and a faster rate of inactivation for the adult isoform compared to the fetal one\textsuperscript{10, 31, 32}. In our study, the increased fraction of adult isoform in hiPSC-CMs upon prolonged culture was paralleled by a faster rate of inactivation, consistent with these previous comparisons of the fetal and adult isoform. However, we did not observe a hyperpolarizing shift in voltage dependence of activation upon prolonged culture. Furthermore, we observed a marked increase in sodium current density with prolonged culture, a feature that was observed for the “adult” versus “fetal” isoform in one of the heterologous expression studies\textsuperscript{32}. Discrepancies between our observations and previous observations in heterologous systems could be related to fundamental differences between the studied...
cell types, i.e. HEK cells or oocytes versus hiPSC-CMs. The former cells lack the complete profile of Na\textsubscript{1.5}-interacting proteins, such as beta-subunits, which could result in different biophysical effects in hiPSC-CMs compared to HEK cells. Moreover, in hiPSC-CMs, a mix of the “adult” and “fetal” isoform is still present upon prolonged culture, therefore the observed properties result from both isoforms, as opposed to heterologous cells, in which only “fetal” or “adult” Na\textsubscript{1.5} is expressed. Alternatively, in hiPSC-CMs, expression of modifiers of Na\textsubscript{1.5} could change in parallel with the increased expression fraction of the “adult” SCN5A isoform during prolonged culture periods. Further studies are required to elucidate the cause of these discrepancies.

As the I230T mutation is located in exon 6, the implications of alternate exon 6/6a usage for disease modeling are intuitive. As the expression fraction of “adult” SCN5A is low, the effects of the mutation are masked by the presence of “fetal” SCN5A in which the mutation is not present. However, also for mutations located in other regions of the gene, the isoform usage can be of critical importance. For example, it was previously demonstrated that the L409P SCN5A mutation, located in exon 10, evoked different effects when present in the “fetal” SCN5A isoform compared to the “adult” isoform, explaining the severe phenotype that was found in utero in that particular patient\textsuperscript{31}. This underscores the fact that the consequence of isoform usage on mutation severity cannot be predicted easily, and should therefore always be taken into consideration in studies aimed to elucidate the phenotypic effects of mutations related to the cardiac sodium channel function. Apart from affecting the interpretation of the biophysical channel defect associated with a given mutation and its relation to the patient phenotype, high expression fractions of the “fetal” SCN5A isoform in hiPSC-CMs could affect the channel sensitivity to Na\textsubscript{1.5}-blocking drugs. This has been demonstrated for SCN1A, which encodes a sodium channel that is mainly present in neuronal tissue. SCN1A is alternatively spliced similarly to SCN5A, i.e. in a “fetal” and “adult” isoform that differ in the usage of a mutually exclusive spliced exon, and it was demonstrated that sensitivity to antiepileptic drugs depends on the isoform that is expressed\textsuperscript{35}. All hiPSC-CM cell lines we studied showed a relative increase in usage of the “adult” isoform with prolonged culture, in line with the time-dependent maturation of the cells. Nevertheless, the fraction of “adult” SCN5A that is present in adult cardiomyocytes, in which expression of “fetal” SCN5A is negligible, was not reached in any of the analysed lines. This observation highlights the fact that in hiPSC-CMs the maturation state that is reached after prolonged culture is still incomplete. Extending the culture time to even longer periods might further stimulate the expression of the “adult” SCN5A isoform. However, previous studies demonstrated that the phenotype of hiPSC-CMs was still relatively immature compared to adult cardiomyocytes even after a culture period for up to one year\textsuperscript{28}. It remains to be determined whether other strategies that are aimed at improving the maturation state of hiPSC-CMs (e.g. electrical stimulation or mechanical
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At short-term culture the two hiPSC-CMs lines harboring the I230T mutation (I230T\textsuperscript{het} and I230T\textsuperscript{homo}) contained a higher ‘starting’ fraction of adult SCN5A isoform compared to the two control lines (Ctrl1 and Ctrl2), with the I230T\textsuperscript{homo} line having the highest fraction. Although these differences could relate to line-to-line variability, we postulated that since the mutation occurred within a splice enhancer motif, these differences may also, at least in part, be due to the effect of the mutation on splicing efficiency of exon 6. This hypothesis is supported by the minigene assay we conducted which clearly demonstrated that the nucleotide change associated with the mutation promoted the inclusion of adult exon 6. In the minigene assay, the usage of the “adult” exon did not occur at the expense of the “fetal” one, as opposed to the hiPSC-CM system, in which the splicing of exon 6 and exon 6a is mutually exclusive. This likely indicates that the system used for the assay lacked certain splice factors that are required for splicing in a mutually exclusive manner, or that genomic information was missing from the minigene construct.

The present study was originally initiated to refine our understanding of the pathophysiological mechanism of a recessive form of SCN5A-related conduction disorder by generating and analysing hiPSC-CMs from a heterozygous and homozygous I230T mutation carrier. Our electrophysiological data obtained in the late-stage hiPSC-CMs demonstrate loss of sodium channel function effects in line with the clinical presentation among carriers and suggests an intermediate loss of \( I_{\text{Na}} \) in the heterozygous state compared to the homozygous state. The predicted severe loss of \( I_{\text{Na}} \) in homozygous carriers is in line with the severe and early clinical phenotype in these patients. Yet, in spite of the predicted loss of sodium channel function, heterozygous patients did not suffer arrhythmia and had normal ECGs. This refractoriness suggests protective mechanisms such as conduction reserve. Indeed, other such cases in which even a predicted 50% reduction in \( I_{\text{Na}} \) is tolerated (e.g. the mutation W156X\textsuperscript{36}) have been described. Conversely, the fact that mutations with effects on \( I_{\text{Na}} \) of similar severity are found in heterozygous carriers who are symptomatic\textsuperscript{37,38}, suggests that other factors, extrinsic from \( I_{\text{Na}} \), may also contribute to the disease.

In human heart, the fetal splice isoform of SCN5A is predominantly expressed before birth and is gradually replaced by the adult isoform\textsuperscript{31}. As a result, mutations that are present in ‘adult’ exon 6 may be tolerated up to the stage where the adult isoform predominates. This may explain why patients carrying the homozygous I230T mutation survive whereas one would perhaps expect a highly severe, if not lethal, phenotype taking into account the degree of sodium channel loss-of-function that is predicted by our findings. In this respect it is interesting to note that among the homozygous or compound heterozygous SCN5A mutations that have been published\textsuperscript{36,39–42}, there
appears to be a preponderance of mutations (4 out of 8) in exon 6 (R225W\textsuperscript{26}, T220I\textsuperscript{41}, A226V\textsuperscript{42} and the I230T mutation presented here), supporting the idea that mutations in this exon are more tolerated as compared to others in the homozygous or compound heterozygous state. As for the I230T mutation, it could be speculated that by virtue of its effects on ‘adult’ exon 6 usage alongside its effects on sodium channel function, patients carrying this mutation may have symptoms at a younger age than in patients harboring mutations in exon 6 that do not affect splicing. Considering the rarity of disease-causing mutations in this exon and the fact that the disease phenotype could be affected by more than sodium channel function alone, these hypotheses are difficult to investigate.

In our study we derived cell lines from four individuals, each with a different genetic background. We therefore cannot exclude that differences we observed between the control and mutant lines may also arise from other genetic variation. Another limitation of our study is that we only studied one individual per genotype (i.e. no biological replicates were studied for the homozygous and heterozygous states) and that only two control individuals were studied. Yet, a number of factors support the notion that the differences we observed in $I_{\text{Na}}$ and action potential characteristics are largely due to the mutation rather than genetic background differences. One of these is the fact that we studied two different control lines derived from unrelated individuals that had electrophysiological characteristics that were similarly different from the two mutant lines. Furthermore, the two mutant lines were derived from first-degree relatives who share 50% of their genome thereby reducing genetic background effects. Finally, the intermediate effects that we observed in the heterozygous line compared to the homozygous line are concordant with the expected dosage effect of the mutation across the two lines.

In conclusion, our study demonstrates that expression fractions of the adult and fetal isoforms of $\text{SCN5A}$ in hiPSC-CMs depend on the time in culture and affect the phenotypic assesment of mutations in these cells. This new aspect of electrophysiological immaturity should be taken into account in studies focussing on the effects of $\text{SCN5A}$ mutations in hiPSC-CMs.

\textbf{Acknowledgements}

We thank Dr. Jan Ruijter for his valuable insights and advice related to the qPCR experiments. We acknowledge Professor Jolanda van der Velden and Emeritus Professor Cristobal Guillermo Dos Remedios from the Sydney Human Heart Tissue Bank for providing the human heart samples.
REFERENCES


## Supplementary Materials

### Supplementary Tables

**Table S1.** List of the applied primers used for PCR per gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’ to 3’</th>
<th>Reverse primer 5’ to 3’</th>
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<td>DPPA4</td>
<td>TGGGTTCAGGTGGTGTTGG</td>
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<td>ZNF206</td>
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</table>

**Table S2.** Average cell capacitance and series resistance in all experimental groups. Values are depicted as mean±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Early-stage hiPSC-CMs</th>
<th>Late-stage hiPSC-CMs</th>
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<td>Ctrl1</td>
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<td><strong>Cell capacitance (pF)</strong></td>
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<td><strong>Series resistance (MΩ)</strong></td>
<td>6.1±0.3</td>
<td>6.6±0.5</td>
</tr>
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</table>
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**Supplementary Figures**

**Experimental scheme for electrophysiological measurements**

Figure S1. Scheme of experimental approach depicting the different time points during differentiation at which the different steps, i.e. addition of lactate, enzymatic dissociation, collection of RNA and electrophysiological measurements, were performed.
Figure S2. Comparison of \( I_{\text{Na}} \) properties in control 1 (Ctrl1) hiPSC-CMs measured after a short and extended culture period (early- and late-stage, respectively). Current-voltage relationships (A), voltage dependence of activation (B), voltage dependence of inactivation (C), recovery from inactivation (D) and time dependence of inactivation (E) are shown. Inset in D depicts the voltage clamp protocol to determine P2/P1 values. In E, left and right panel indicate \( \tau_{\text{slow}} \) and \( \tau_{\text{fast}} \), respectively. Upon extended time in culture, \( I_{\text{Na}} \) density, recovery rate and inactivation rate increase. *indicates \( p < 0.05 \) (Mann-Whitney U test).
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Figure S3. Comparison of $I_{\text{Na}}$ properties in control 2 (Ctrl2) hiPSC-CMs measured after a short and extended culture period (early- and late-stage, respectively). Current-voltage relationships (A), voltage dependence of activation (B), voltage dependence of inactivation (C), recovery from inactivation (D) and time dependence of inactivation (E) are shown. Inset in D depicts the voltage clamp protocol to determine $P_2/P_1$ values. In E, left and right panel indicate $\tau_{\text{slow}}$ and $\tau_{\text{fast}}$, respectively. Upon extended time in culture, $I_{\text{Na}}$ density and the slow component of inactivation rate increase, while voltage dependence of inactivation displays a positive shift of 3 mV. *indicates $p < 0.05$ (Mann-Whitley U test).