Human iPSC-derived cardiomyocytes cultured in 3D engineered heart tissue show physiological upstroke velocity and sodium current density

Marc D. Lemoine1,2,3, Ingra Mannhardt2,3, Kaja Breckwoldt2,3, Maksymilian Prondzynski2,3, Frederik Flenner2,3, Bärbel Ulmer2,3, Marc N. Hirt2,3, Christiane Neuber2,3, András Horváth2,4, Benjamin Kloth5, Hermann Reichenspurner5, Stephan Willems1,3, Arne Hansen2,3, Thomas Eschenhagen2,3 & Torsten Christ2,3

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) are a promising tool for drug testing and modelling genetic disorders. Abnormally low upstroke velocity is a current limitation. Here we investigated the use of 3D engineered heart tissue (EHT) as a culture method with greater resemblance to human heart tissue in comparison to standard technique of 2D monolayer (ML) format. INa was measured in ML or EHT using the standard patch-clamp technique. INa density was ~1.8 fold larger in EHT (−18.5 ± 1.9 pA/pF; n = 17) than in ML (−10.3 ± 1.2 pA/pF; n = 23; p < 0.001), approaching densities reported for human CM. Inactivation kinetics, voltage dependency of steady-state inactivation and activation of INa did not differ between EHT and ML and were similar to previously reported values for human CM. Action potential recordings with sharp microelectrodes showed similar upstroke velocities in EHT (219 ± 15 V/s, n = 13) and human left ventricle tissue (LV, 253 ± 7 V/s, n = 25). EHT showed a greater resemblance to LV in CM morphology and subcellular NaV1.5 distribution. INa in hiPSC-CM showed similar biophysical properties as in human CM. The EHT format promotes INa density and action potential upstroke velocity of hiPSC-CM towards adult values, indicating its usefulness as a model for excitability of human cardiac tissue.

Animal-heart tissue is commonly used as a model for human-heart tissue, but exhibits a significantly different action potential (AP) duration and shape, due to different ion channel contributions, interactions and regulation. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) have the great advantage of generating human-like AP-duration and shape. In addition, hiPSC-CM represent a theoretically unlimited source of CM, lacking the ethical concerns that come along with sacrificing animals. The recent progress in the development and generation of hiPSC provides great opportunities to study individualised cardiac electrophysiology, focusing on genetic disorders1 and individualised drug treatment2. However, there are concerns about the immaturity of hiPSC-CM3. One important difference relates to AP upstroke-velocity which, in initial publications, was found to be markedly lower (~2–50%) in hiPSC-CM4–6 than in adult CM7. These findings suggest lower sodium current (INa) density in hiPSC-CM, which is of great physiological importance as INa determines excitability, conduction velocity, refractoriness and triggered activity. Furthermore, INa is an established drug target for antiarrhythmic therapy (flecainide, propafenone, amiodarone, vernakalant and ranolazine). Recent improvements have been

1Department of Cardiology-Electrophysiology, University Heart Center, Hamburg, Germany. 2Department of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. 3DZHK (German Center for Cardiovascular Research), partner site Hamburg/Kiel/Lübeck, Hamburg, Germany. 4Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged, Szeged, Hungary. 5Department of Cardiovascular Surgery, University Heart Center, Hamburg, Germany. Correspondence and requests for materials should be addressed to M.D.L. (email: m.lemoine@uke.de) or T.C. (email: t.christ@uke.de)
brought about by co-culture with non-cardiac cells, long-term culture, hormone stimulation, continuous field stimulation, and variation of substrate stiffness, revealing upstroke velocities of up to 147 V/s. While these values approach the expected range (200–300 V/s) for human adult ventricular tissue, differences remain and a head-to-head comparison under same conditions is lacking.

An alternative approach to increase the maturation of hiPSC-CM is cardiac tissue engineering. CM in hydrogel-based engineered heart tissue (EHT) form a synchronously beating syncytium, which generates contractile force by rhythmically deflecting the two elastic silicone posts it is attached to and thereby performs auxotonic contractile work. Morphological and functional evidence suggest that hiPSC-CM reach a higher degree of maturity in EHT, but electrophysiological data are lacking. Here we directly compared upstroke velocity in hiPSC-CM cultured in 3D (EHT) and in human heart tissue biopsies obtained during the implantation of left ventricular assist devices (LVAD) or heart transplantation, and studied $I_{Na}$ properties in hiPSC-CM from 2D monolayers (ML) and EHT under the conditions published for human adult CM.

**Results**

**Cell capacitance and sodium current.** We compared the $I_{Na}$ of EHT with standard 2D ML using the whole-cell patch clamp technique. Cell size as measured by the cell capacitance showed no statistically significant difference between EHT and ML (Fig. 1A): EHT $28.2 \pm 2.0$ pF ($n = 37$) vs. ML $23.3 \pm 1.9$ pF ($n = 38$). We studied $I_{Na}$ at a reduced extracellular Na concentration, in order to ensure good voltage control and comparability to

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**Figure 1.** Cell capacitance and sodium current. (A) Scatter plot of cell capacitance in hiPSC-CM (mean values in Table 1). (B) Family of original Na current traces elicited by the protocol shown in the inset. (C) Scatter plot of $I_{Na}$ density in hiPSC-CM for voltage-clamp pulse to $-30$ mV from a holding potential of $-110$ mV (EHT vs ML: ***p < 0.001). (D) Correlation of $I_{Na}$ amplitude and cell capacitance. Best fit values for slope: ML $9.7 \pm 2.4$ pA/pF vs. EHT $16.9 \pm 5.9$ pA/pF. Deviation from zero slope was significant for ML (p < 0.001) and EHT (p < 0.05).
previous studies on human adult CM. As expected, current amplitude showed a proportional relation to cell size (Fig. 1D). Mean I_{Na} density was remarkably higher (~80%) in EHT (−18.5 ± 1.9 pA/pF; n = 17) than in ML (−10.3 ± 1.2 pA/pF; n = 23; p < 0.001; Fig. 1C). I–V curves (Fig. 2A) show that the I_{Na} was activated around −55 mV and peaked at −30 mV in EHT and ML. EHT hiPSC-CM showed higher I_{Na} density than ML over the entire activation range (p < 0.05, Fig. 2A). Thus, EHT showed increased I_{Na} density in comparison to ML. To test for a late sodium current, we measured currents at the end of our test-pulse. Currents amounted to −68.9 ± 13.9 pA under control condition and to −70.1 ± 13.9 pA after the application of 30 µM tetrodotoxin (TTX; n = 10, ns, paired t-test). Therefore, we did not find evidence for a persistent/late I_{Na}.

Inactivation, activation and recovery from inactivation. The contribution of I_{Na} to the electrical activity of CM depends critically on the voltage and time-dependent activation and inactivation. Activation curves were calculated from individual I–V curves by normalising peak current amplitudes for their actual driving force and a Boltzmann function was fitted to the data set (Fig. 2C). Voltages for the half-maximum activation (V_{0.5act}) of I_{Na} and curve steepness (k_{act}) did not differ between EHT and ML (Table 1). In order to characterise the inactivation kinetics of I_{Na}, we fitted a single exponential function to current traces at different test pulse potentials (Fig. 2B). Time constants were shorter at increasing voltages of the test pulse without differences between EHT and ML.

In general, resting membrane potential (RMP) in CM is less negative than V_{0.5inact}. Therefore, only a minority of cardiac Na channels can be activated. Even small changes in RMP have strong effects on Na channel availability. We simulated different RMPs by applying variable conditioning pre-pulses from −120 mV to −30 mV for 1000 ms to determine steady-state inactivation. The mean data revealed no differences between EHT and ML in
transcript levels of the low-TTX-sensitive cardiac isoform NaV 1.5 (SCN5A) were predominant without difference even within the cell. NaV 1.5 was distributed in the Z-disks and in the intercalated disks of adult cardiac tissue (Fig. 5B) and to the literature (Table 2). AP upstroke velocity in EHT did not differ significantly from that recorded in LV tissue (EHT: 219 ± 6 mV/s, n = 13, N = 6 vs. LV: 253 ± 16 mV/s, n = 25, N = 5; ns; Fig. 4A and B). Maximum diastolic potential (MDP), RMP immediately before the upstroke (= take-off potential) and AP amplitude (Fig. 4B) also did not differ significantly between EHT (MDP: −78.4 ± 2.9 mV, RMP: −73.5 ± 1.6 mV, 102.7 ± 2.8 mV, n = 13, N = 6) and LV (MDP: −74.8 ± 1.1 mV, RMP: −74.8 ± 1.1 mV, APA: 104.8 ± 1.4 mV, n = 25, N = 5).

Cell structure and subcellular distribution of NaV1.5. HiPSC-CM in EHT were oriented in parallel and showed a rod-shape morphology with sarcomere alignment comparable to LV tissue (Fig. 5). In contrast, hiPSC-CM in ML format showed an increased circularity and the sarcomeres were oriented in different directions even within the cell. NaV1.5 was distributed in the Z-disks and in the intercalated disks of adult cardiac tissue (Fig. 5). The subcellular distribution of NaV1.5 in ML hiPSC-CM was markedly different. It shows perinuclear enhancement with less pronounced signalling at the cell periphery and without co-localisation with α-actinin or enrichment at cell-cell contacts similar to what has been found in previous publications. In contrast, hiPSC-CM in EHT showed a more pronounced expression of NaV1.5 in the periphery of the CM. Few CM in EHT showed co-localisation of NaV1.5 to Z-disks (arrowheads in Fig. 5B) and enhanced expression of

|                       | ML n = 28.2 ± 2.0 | EHT n = 18.5 ± 1.9 | p-value |
|------------------------|------------------|-------------------|---------|
| V_{m} (mV)             | −34.6 ± 2.1      | −36.2 ± 0.7       | 0.353   |
| k_{inact}              | 5.8 ± 0.2        | 6.2 ± 0.2         | 0.187   |
| Recovery of inactivation | 10 ± 11         | 8 ± 8             |         |
| Proportion fast (%)    | 54.7 ± 14.1      | 49.7 ± 6.3        | 0.376   |
| r_{fast} (ms)          | 5.4 ± 1.3        | 6.7 ± 1.3         | 0.488   |
| r_{slow} (ms)          | 93.4 ± 17.3      | 90.4 ± 9.0        | 0.857   |

Table 1. Biophysical parameters of ML and EHT cultured hiPSC-CM. HiPSC-CM: human induced pluripotent stem cell-derived cardiomyocytes; n: number of cardiomyocytes; I_{Na} density measured at −30 mV from −110 mV holding potential; V_{m,50} voltage of half-maximal (in)activation; k: slope factor of voltage-dependence of (in) activation; τ_{fast} / τ_{slow} fast and slow time constants of recovery from inactivation. Values are mean ± SEM.
Na\textsubscript{v}1.5 at cell-cell contacts orthogonal to the CM orientation (arrow in Fig. 5B), comparable to the intercalated disk of adult cardiac tissue. Proper impulse propagation does not only depend on the sodium channels, but also on polarized connexin-43 expression. We found pronounced connexin-43 staining at the cell membranes of hiPSC-CM in EHT, but no clear enhancement at end-to-end over lateral cell-cell contacts typical of adult human LV (Supplementary Figure 3).

### Discussion

In the present study, we investigated whether culture of hiPSC-CM in the EHT format leads to a higher resemblance with adult human CM in terms of $I_{Na}$ density, upstroke velocity, CM morphology and subcellular distribution of Na\textsubscript{v}1.5.

$I_{Na}$ densities are difficult to compare among different studies, as experimental conditions differ widely with respect to temperature and Na concentrations (as listed in Table 2). Here we used the same experimental conditions used previously to measure $I_{Na}$ in human adult ventricular CM\textsuperscript{17}, allowing for reliable comparisons with hiPSC-CM. Due to the limited availability of human adult ventricular tissue, studies analysing its electrophysiological properties are rare. To our knowledge, only one publication has studied $I_{Na}$ in human adult ventricular CM\textsuperscript{18} reporting an $I_{Na}$ density of $-20.2$ pA/pF. Human atrial CM $I_{Na}$ density measured under the same conditions amounted to $-17.8$ pA/pF; others reported values between $-4$ and $-30$ pA/pF\textsuperscript{17,21,22}. Thus, the $I_{Na}$ densities in EHT hiPSC-CM ($-18.5$ pA/pF) fit nicely with ventricular and atrial adult CM. The observation that $I_{Na}$ density in EHT CM was nearly two fold higher than CM cultured in conventional ML ($-10.7$ pA/pF) provides evidence for...
the hypothesis that EHT culture improves the maturity of CM. The isometric mode of contraction of hiPSC-CM cultured on rigid surfaces (ML) in comparison to the auxotonic mode of contraction with defined load (EHT) might cause the difference in $I_{\text{Na}}$ density. This supports recent data from hiPSC-CM cultured on soft substrate\textsuperscript{11, 12}, suggesting that auxotonic work is essential for the development of proper $I_{\text{Na}}$ density. $I_{\text{Na}}$ density in mammalian CM increases constantly during cardiac development from embryonic to neonatal stages until the adult state\textsuperscript{23–25}, which occurs in parallel with increasing SCN5A expression during differentiation and culture of hiPSC-CM (Supplementary Figure 2 and Fig. 3B). Thus, the increase in $I_{\text{Na}}$ density might be part of the maturation process.

In order to elucidate potential mechanisms causing the greater $I_{\text{Na}}$ density in EHT vs. ML, we reanalysed our previously published transcriptome of hiPSC-CM\textsuperscript{15}. We focused on the expression of genes thought to influence $I_{\text{Na}}$. On the one hand, we found lower mRNA levels in EHT for the transforming growth factor beta 1 ($\text{TGFB1}$) which is a multifunctional cytokine and may reduce $\text{NaV1.5}$ expression\textsuperscript{26}. On the other hand, we found higher mRNA levels in EHT of proteins believed to enhance $I_{\text{Na}}$ function: epidermal growth factor ($\text{EGF}$), promoting ubiquitously growth, proliferation and differentiation\textsuperscript{27}; anchoring adaptor ankyrin-G ($\text{ANK3}$)\textsuperscript{28}, which is involved in ion channel trafficking to the cell membrane, and plakophilin-2 ($\text{PKP2}$), which is part of cytoskeleton and cell-cell contact\textsuperscript{29}. Interestingly, missense mutations in plakophilin-2 are known to cause arrhythmogenic right ventricular cardiomyopathy and $I_{\text{Na}}$ deficit\textsuperscript{30}.

The contribution of the short lasting $I_{\text{Na}}$ to the AP also depends on inactivation kinetics, which can be characterised by fitting a single exponential non-linear curve to the negative downslope of the $I_{\text{Na}}$. Inactivation kinetics revealed similar time constants and voltage-dependency in ML and EHT (Fig. 2B) fitting nicely to data reported for human adult CM\textsuperscript{21}. The voltage-dependency of activation and steady-state inactivation were indistinguishable between EHT and ML (Fig. 2C), implying that the higher $I_{\text{Na}}$ density in EHT may be explained by a greater number of functional Na channels. As experimental conditions like time after rupture and temperature are known to shift $V_{0.5\text{act}}$ and $V_{0.5\text{inact}}$ to approximately the same extent, we calculated the actual difference (delta) between $V_{0.5\text{act}}$ and $V_{0.5\text{inact}}$. We found values of 55.1 mV and 55.3 mV for EHT and ML, respectively (Table 2), which are comparable to published data\textsuperscript{16, 17, 21} for adult CM (54–58 mV). This finding is in contrast to previous measurements in hiPSC-CM cultured in ML showing markedly smaller values\textsuperscript{5, 12, 22}, including a recent study that describes the culturing of hiPSC-CM on a soft substrate\textsuperscript{12}. It is unclear if these discrepancies relate to technical differences or to intrinsic properties of different cell lines. Overall, we conclude that EHT format does not affect the voltage-dependent steady-state inactivation and activation in hiPSC-CM and these parameters are similar in our hiPSC-CM to that in human adult CM.

The difference (delta) between $V_{0.5\text{act}}$ and $V_{0.5\text{inact}}$ mentioned above is not trivial: more overlap should generate more window current. Previously published studies on hiPSC-CM\textsuperscript{5, 12, 22} have shown reduced differences (delta) between $V_{0.5\text{act}}$ and $V_{0.5\text{inact}}$, and as a consequence a higher calculated window current (4–12%, Table 2). Larger window currents may partly explain persistent/late $I_{\text{Na}}$ in CM and might have a critical influence on AP duration\textsuperscript{11}. In our experiments with hiPSC-CM, we found only a small amount of window current as in adult CM and no persistent/late $I_{\text{Na}}$. At the maximum degree of overlap (inset Fig. 2C), $I_{\text{Na}}$ in hiPSC-CM showed similar fractions of maximal availability and conductance (1.4% and 1.8% for EHT and ML, respectively) as human adult CM (~2% and ~1% for atrial\textsuperscript{17} and ventricular CM\textsuperscript{16}, respectively). It should be noted that the amount of window current might be an additional marker in the maturation process, since window currents decrease during the development of chick embryonic CM\textsuperscript{32}. 

Figure 4. Action potential characterisation. (A) Example of action potentials (AP) and the AP upstroke velocity (inset) measured in human induced pluripotent stem cell-derived cardiomyocytes cultured in engineered heart tissue (EHT) or in human left ventricular tissue (LV) at 36.5 °C paced at 1 Hz. (B) Corresponding AP parameters. N: number of EHTs/LV tissues; n: number of impalements with the sharp microelectrode. RMP, resting membrane potential; APA, action potential amplitude; dV/dt, maximum upstroke velocity; APD_{90}, AP duration at 90% repolarisation.
Table 2. Comparison of INa properties in isolated human induced pluripotent stem cell-derived and adult cardiomyocytes. HiPSC-CM: human induced pluripotent stem cell-derived cardiomyocyte; ML: monolayer; EHT: engineered heart tissue; V0.5: voltage of half-maximal (in)activation; k: slope factor of voltage-dependence of (in)activation; *overlap-potential (Voverlap) = (V0.5Inact − V0.5act)/(kInact) (details in supplementary data); †availability at overlap (%) = calculated = 1/(1 + EXP((V0.5Inact − overlap-potential)/kInact)) * 100; INa ext: sodium concentration of the extracellular (bath) solution; INa int: sodium concentration of the intracellular (pipette) solution; MDP: maximum diastolic potential; RMP: resting membrane potential (=take-off potential); dV/dtmax: maximum upstroke velocity; APA: action potential amplitude.

| CM-type | ML hiPSC | EHT hiPSC | ventricular | ventricular | atrial | atrial | atrial | atrial | atrial | atrial | ML hiPSC (on Matri-gel) | Single hiPSC | ML hiPSC | ML hiPSC | ML hiPSC |
|---------|----------|-----------|-------------|-------------|--------|--------|--------|--------|--------|--------|--------------------------|-------------|-----------|-----------|-----------|-----------|
| Capacitance (pF) | 24.1 | 27.9 | 194 | 126 | 73.1 | 72.1 | 66 | 89 | 17.0 | 42 | 15.8 | |
| Peak INa density (pA/pF) | −10.3 | −18.5 | −20.2 | −17.8 | −14 | −30 | −50.2 | −37 | −30.0 | −160 | −105 | − −68 | −118 | −264.4 | −216.7 |
| V0.5 activation (mV) | −34.6 | −36.2 | −42.8 | −38.9 | −38.8 | −50.2 | −38.6 | −44 | −42.4 | −42 | −34.1 | |
| kInact | 5.8 | 5.9 | 6.0 | 6.5 | 5.3 | 7.2 | 1.8 | 5.9 | | | |
| V0.5 inactivation (mV) | −89.8 | −91.3 | −97.3 | −95.8 | −97.1 | −97.2 | −95.1 | −72.2 | −77 | −88.0 | −82.8 | −61.4 | −72.1 | |
| %overlap-potential | 6.1 | 7 | 5.8 | 5.3 | 6.2 | 7.4 | 4.9 | 7.6 | 5.7 | | |
| Temperature (°C) | 21 | 21 | 17 | 17 | 23 | 21 | 22 | 24 | 21 | 37 | 22 | 24 | 36 | 36 |
| INa ext (mmol/L) | 5 | 5 | 5 | 5 | 5 | 5 | 10 | 120 | 150 | 20 | 10 | 135 | 130 | 50 | |
| INa int (mmol/L) | 5 | 5 | 5 | 5 | 5 | 5 | 10 | 70 | 10 | 5 | 5 | 5 | 5 | 10 | |
| Holding potential (mV) | −110 | −110 | −140 | −140 | −120 | −110 | −140 | −135 | −120 | −140 | −120 | −90 | −90 | −80 | |
| Pulse frequency (Hz) | 0.5 | 0.5 | 0.1 | 0.1 | 0.1 | 0.5 | 0.1 | 0.5 | 0.2 | | |
| INa TTX (µmol/L) | 1.4 | 1.7 | 1.1 | 10.6 | 0.6 | | | | | | |
| Temperature (°C) for AP | 37 | 37 | 37 | 37 | 22 | 22 | 24 | 21 | 37 | 22 | 24 | 36 | 36 | |
| MDP (mV) | −78.4 | −74.8 | −72.6 | −77.5 | 74.9 | −60.9 | −72.4 | −75.6 | |
| RMP (=take-off) (mV) | −73.5 | −74.8 | −72.6 | −70.5 | | | | | |
| dV/dtmax (V/s) | 219 | 253 | 230 | 146.5 | 84 | 13.1 | 115.7 | 27.8 | |
| APA (mV) | 102.7 | 104.8 | 94.3 | 116 | 124 | 88.1 | 106.0 | 104.0 | |

Interestingly, not only does the RMP influence INa density, but also vice versa, INa may influence RMP. Window INa may contribute significantly to RMP, especially when inward rectifier currents are small, as in human atrial trabeculae. Imanishi et al. have shown that high concentrations of TTX hyperpolarized the membrane potential of quiescent human atrial trabecular by about 7 mV. Consequently, window INa may be of particular relevance in hiPSC-CM, as less negative RMP and small inwardly rectifying potassium currents are consistently reported for hiPSC-CM.

Sodium currents are known to recover quickly from voltage-dependent inactivation. Recovery from inactivation critically determines refractoriness and influences the susceptibility to tachyarrhythmia. In human adult atrial and ventricular CM recovery from inactivation could be fitted by a two-time constant function when plotting peak currents against different recovery time intervals. Earlier studies on hiPSC-CM from ML reported similar characteristics of recovery from inactivation as here. It should be noted that both this study and Ma et al. found a somewhat faster recovery from inactivation in hiPSC-CM than in human adult CM. Whether this small difference has physiological relevance needs to be elucidated by computer modelling and functional studies.

We used TTX-inhibition of INa to evaluate whether non-cardiac isoforms contribute significantly to INa in hiPSC-CM, which might be a sign of immaturity (Supplementary Figure 2). In hiPSC-CM, the effect size for
low nanomolar concentrations of TTX fits perfectly to a single-site binding model with low sensitivity (IC$_{50}$ 1.3 µmol/L). Therefore, we would not assume relevant contribution from highly sensitive isoforms of Na channels to peak currents. Published TTX-sensitivities in atrial and ventricular CM amount to 1.1 µmol/L$^{17}$ and 1.7 µmol/L$^{16}$ showing that the TTX-sensitivity of $I_{Na}$ in hiPSC-CM was similar to that in adult CM. Quantitative evaluation of the transcript levels of different Na channel isoforms confirmed that expression was dominated by the expression of the low-TTX sensitive isoform $Na_{V}$ 1.5 (SCN5A) compared to the highly-TTX sensitive neuronal isoforms $Na_{V}$ 1.1–1.3, 1.6 (SCN1–3A, SCN6A). The TTX-resistant neuronal isoform $Na_{V}$ 1.8 (SCN10A) was expressed at intermediate levels and showed lower absolute transcription levels in hiPSC-CM than in LV.

To the best of our knowledge, we show here for the first time AP in hiPSC-CM with an upstroke velocity similar to human adult ventricular tissue (200–300 V/s). Previous studies in hiPSC-CM have reported heterogeneous and overall lower upstroke velocities (Table 2) at ~40 V/s for ventricular-like AP in isolated cells$^{4,6,22}$ or in embryoid bodies$^{5}$. Recent approaches to culture hiPSC-CM on a soft substrate of extracellular matrix as single CM$^{11}$ or ML$^{12}$ revealed higher $I_{Na}$ density$^{11,12}$ and higher upstroke velocity of 147 V/s$^{12}$ in comparison to cultures on a stiff substrate (65 V/s). Collectively, the data indicate that culture conditions allowing auxotonic contractions of hiPSC-CM against a flexible resistance increases the resemblance in $I_{Na}$ density known for adult CM. This will

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**Figure 5.** Immunofluorescence analysis. Subcellular localisation of $\alpha$-actinin (green), $Na_{V}$ 1.5 (red) and nuclei (blue) in a whole mount immunofluorescent confocal section of left ventricular tissue (LV, two examples, A) and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) in engineered heart tissue (EHT, two examples, B) and monolayer (ML, C). In contrast to ML, EHT showed a parallel orientation of CM, a more rod-shaped morphology, sarcomere alignment and $Na_{V}$ 1.5 enhancement at cell-cell contact (arrow). In some parts of the EHT, $Na_{V}$ 1.5 was co-localised with $\alpha$-actinin at Z-disks (see arrowheads in inset with 2.5 fold magnification), comparable to LV. Scale bar for all images is 10 µm. Black rectangles were placed aside confocal images in A for symmetrical appearance.
be important for the further use of hiPSC-CM in drug testing and modelling of genetically determined cardiac diseases.

We also found that AP in hiPSC-CM in EHTs showed a steeper early repolarisation and were considerably shorter than APs derived from LV tissue. A possible explanation is that APs from human tissue were all recorded from the subendocardial myocardium, which is known to express much less transient outward potassium current (Ito) and therefore exhibits longer APs than subepicardial regions35. Future work is warranted to evaluate whether APs in EHT are in fact ‘subepicardial-like’ or whether the observed difference indicate a specific hiPSC-CM phenotype or a peculiarity of the cell line under investigation.

Immunohistochemistry revealed a parallel orientation of CM with a more rod-shaped morphology and sarcomere alignment of hiPSC-CM than in ML (Fig. 5). Interestingly, the subcellular distribution of NaV1.5 in EHT hiPSC-CM showed an enhancement of NaV1.5 at cell-cell contacts and, in some CM, pronounced signals, similar to those in the intercalated disks of the LV. Since NaV1.5 relocates from lateral to intercalated disks during cardiac development36, the enhancement of NaV1.5 in the direction of sarcomere orientation might be another hint for structural maturation of hiPSC-CM by the EHT format. Additionally, some hiPSC-CM in EHT showed co-localisation of NaV1.5 with α-actinin at Z-disks, while hiPSC-CM in ML did not, as shown previously19.

Although the EHT format facilitated structural maturation, the cell size of hiPSC-CM was similar to those in ML format (~25 µm²) and much smaller in comparison to adult LV CM (~100–200 µm²)12,37. As cell size increases during the embryonic development of cardiomyocytes37, the small cell size may indicate an early stage of development. Smaller cells have a greater membrane area to volume ratio, but the physiological relevance of this remains unclear. From a technical point of view, smaller cells should conduct smaller absolute membrane currents making patch-clamp studies more technically demanding16.

A limitation of this study is that all LV tissue was obtained from patients with advanced heart failure due to dilated cardiomyopathy, since access to living non-failing heart tissue is virtually impossible. We therefore cannot fully exclude electrophysiological differences to healthy tissue. However, previous publications have shown no differences in sodium current properties from failing or non-failing hearts17 and upstroke velocity in our hands were similar to values reported for non-failing human heart18.

In conclusion, we have characterized INa in hiPSC-CM in 3D EHT and conventional 2D ML culture and compared AP characteristics in hiPSC-EHTs and human ventricular tissue. The main findings are 1) a higher INa density and a similar upstroke velocity in EHT as in human adult ventricular tissue, 2) similar voltage-dependent inactivation and activation of INa in EHT and ML, 3) no evidence for relevant non-cardiac isoforms contributing to INa in hiPSC-CM and 4) a higher resemblance of hiPSC-CM in EHT to LV concerning structure and subcellular NaV1.5 distribution than ML. Thus, our data suggest that EHT culture of hiPSC-CM may improve the validity of in-vitro experiments studying electrophysiological questions.

Methods
An expanded method section is available in the supplementary data. Human materials and experimental protocols. This investigation conforms to all principles outlined by the Declaration of Helsinki and the Medical Association of Hamburg. According to the guidelines of the ethical review committee of the Medical Association of Hamburg, Germany, there is no need for a specific approval in this case since patient data were used anonymized. All materials from patients were taken with informed consent of the donors. Left ventricular free wall samples were obtained from patients undergoing implantation of left ventricular assist device (LVAD) or heart transplantation.

Generation and culture of human induced pluripotent stem cell-derived cardiomyocytes in engineered heart tissue and monolayer format. As previously described15, single cell suspensions of hiPSC-CM were either subjected to EHT generation in a 24-well format (1x10⁶ hiPSC-CM/EHT in a fibrin matrix (total volume 100 µl) consisting of 10 µl/100 µl Matrigel [BD Bioscience, 256235], 5 mg/ml bovine fibrinogen (200 mg/ml in NaCl 0.9% [Sigma, F4753] plus 0.5 µg/mg aprotinin [Sigma, A1153]), 2x DMEM, 10 µM Y-27632 and 3 U/ml thrombin [Biopur, BP11101104]) or cultured conventionally in ML gelatin-coated 24-well plates (4x10⁵ hiPSC-CM/Well, 2 cm²). Culture media and duration were kept identical. For patch clamp measurements, hiPSC-CM in EHT and ML were isolated with collagenase II (200 U/ml, Worthington, L5001176) after a 24–29 day culturing period, and re-plated on gelatin-coated coverslips for 24–48 h in order to maintain adherence under perfusion.

Patch-clamp experiments. INa recordings were performed as described previously17. In brief, borosilicate glass microelectrode pipettes (tip resistances 1.5–3.0 MΩhm) were used to record INa in whole-cell configuration at room temperature (21 ± 1 °C) with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA). Pipette and bath solution contained 5 mmol/L NaCl.

Action potential measurements. APs were recorded as described previously17 with standard sharp micro-electrodes in intact EHTs (25–60 days old) or LV tissue superfused with Tyrode’s solution at 36.5 ± 0.5 °C field-stimulated at 1 Hz (n = number of total impalements, N = number of EHT/LV tissue).

Immunofluorescence. Immunofluorescence was performed as described previously15. Briefly, EHT or LV tissue were fixed in formaldehyde overnight at 4 °C, blocked for 6 h and incubated with primary antibodies (monoclonal mouse anti-α-actinin; monoclonal rabbit anti-NaV1.5) and secondary antibodies and nuclear staining (Alexa Fluor® 488 goat-anti-rabbit; Alexa Fluor® 546 goat-anti-mouse; DRAQ5TM). 2D cultures were cultivated on 96-well plates and were fixed for 20 minutes at 4 °C and stained accordingly with the exception of using Hoechst 33342 for nuclei staining.
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Author Contributions

M.D.L., A.H., T.E. and T.C. conceived the experiments, M.D.L., I.M., K.B., M.P., F.F., B.U., H.R., M.N.H., C.N., A.H., and B.K. organised or conducted the experiments and acquired data. M.D.L. analysed data. M.D.L., T.E. and T.C. wrote the manuscript. S.W., A.H., T.E. and T.C. acquired financial support for this project. All authors reviewed the manuscript.

Additional Information

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