Human ventricular stem cell cardiomyocytes: validating *in vitro* assays and screening platforms for proarrhythmia risk prediction

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Introduction

Metrion offers high quality cardiac ion channel services to predict human arrhythmia risk, as required by the FDA's Comprehensive in vitro Proarrhythmia Assay (**CiPA**) initiative, including.

- High quality *in vitro* human cardiac ion channel patch clamp assays,
- Comprehensive *in silico* action potential (AP) models
- Predictive phenotypic assays utilising induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM)

To fulfil the last requirement of the CiPA initiative, the suitability and maturity of ventricular iPSC-CM need to be determined. In the current study, two ventricular iPSC-CM cell lines (LDN-1 and LDN-2) were generated and their molecular and biophysical properties compared with a commercial iPSC-CM cell line (COM-1) using three different methodologies:

1. mRNA expression of key cardiomyocyte markers using qRT-PCR (Leiden).
2. Phenotypic measurements of cell contraction (impedance) assessed using the CardioExcyte96 platform (Nanion).
3. Electrophysiology properties were evaluated by manual patch clamp (Metrion)
   - Whole-cell voltage clamp recordings of ionic currents
   - Current clamp measurements of action potential (AP) parameters and pharmacology

Materials and Methods

Cell Lines: Two ventricular iPSC-CM cell lines generated by Leiden University were assessed in this study (LDN-1 and LDN-2) and compared to commercially available ventricular iPSC-CM cell line (COM-1).
Cell differentiation: LDN iPSC-CM (LDN-1 and LDN-2) were differentiated from a common parental cell line using standard monolayer protocols from Leiden University Medical Center and a commercial provider. Beating areas were observed at day 10 and were frozen at day 21. Post-thawing, LDN iPSC-CM were maintained in CM medium (Birket et al, 2015) and refreshed every 3 days.
Molecular biology: After 1 week in culture, cells were lysed for RNA isolation with NucleoSpin® RNA kit (Machery-Nagel). 500 ng RNA was taken for cDNA synthesis using iScript™ cDNA synthesis kit (Bio Rad). Expression of the target genes ACTN2, SCN5A, KCNJ2, KCNH2, CACNA1C and RYR2 were assessed using the Bio-Rad CFX384 Real-Time PCR detection system.
CardioExcyte96: iPSC-CM were seeded at 10 and 30 K cells/well for LDN-1 and COM-1 respectively onto fibronectin-coated NSP-96 Sensor Plates (Nanion Technologies GmbH). Medium was exchanged daily until the conclusion of the assay. Pharmacology experiments were conducted on 5 days after plating for COM-1 cells and 6 days for LDN-1. At least 4 hrs before drug application the medium was completely removed from the wells and 100 µl fresh medium was added. For the experiments, 100 µl of 2x concentration compound solution was added the well to give 1x final concentration. After compound addition, impedance measurements were taken every 5 min for 10 s for the first 60 min and then every 30 min for up to 24 h. Data were analysed using DataControl 96 (Nanion Technologies GmbH).
Manual patch clamp: iPSC-CM cells were seeded onto fibronectin-coated coverslips and cultured at 37 °C (5 % CO_2). AP were recorded 7-10 days after cell seeding at RT in current clamp mode using perforated patch (100 µg/ml gramicidin). For evoked AP cells were paced at 1 Hz with a field stimulator. Voltage clamp recordings were obtained from single cells using the conventional whole-cell patch clamp configuration with protocols and solutions designed to isolate the ionic current of interest (Table 1).

	External				Internal			
	I_{Na}	I_{Ca}	I_K	CC	I_{Na}	I_{Ca}	I_K	CC
NaCl	50	-	140	140	5	5	5	5
TEA-Cl	1	140	-	-	-	-	-	-
KCl	5.4	5.4	5.4	5.4	-	-	20	125
K-Asp	-	-	-	-	-	-	110	-
$CaCl_2$	1.8	1.8	1.8	1.8	-	-	-	-
$MgCl_2$	1	1	1	1	-	-	1	5
CsCl	90	-	-	-	130	130	-	-
Glucose	10	10	10	10	-	-	-	-
HEPES	10	10	10	10	10	10	10	10
MgATP	-	-	-	-	5	5	5	-
$CdCl_2$	0.3	-	0.3	-	-	-	-	-
EGTA	-	-	-	-	10	10	10	5
4-AP	-	2	-	-	-	-	-	-
pH	7.4 CsOH	7.4 CsOH	7.4 NaOH	7.4 NaOH	7.2 CsOH	7.2 CsOH	7.2 KOH	7.2 KOH

Table 1: Manual patch clamp solutions. Composition (in mM) of external and internal solutions used for voltage and current (CC) manual patch clamp experiments.

Data were acquired with EPC10 amplifiers and PatchMaster software (HEKA Elektronik, Germany). Analog signals were low-pass filtered at 10 kHz before digitization at 20 kHz. Spontaneous AP were analysed with CAPA software (SSCE UG, Germany). The AP parameters analysed are shown in Figure 1. In this study the Maximum Depolarisation Rate (MDR) could not be calculated accurately for evoked AP, therefore Time To Peak (TTP) was used by measuring the time (ms) from the end of the stimulation artefact to the AP peak. Data are reported as mean ± SEM.

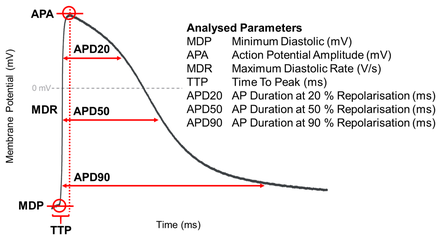


Figure 1: Action potential parameters Example action potential indicating the parameters which are quantified using HEKA FitMaster (evoked AP) and CAPA software (spontaneous AP) in this study.

1. Cardiac gene expression

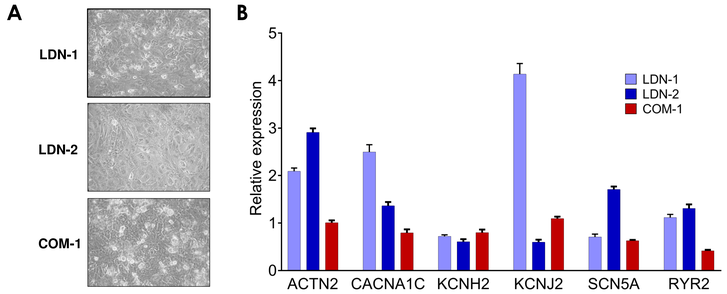


Figure 2: mRNA expression of cardiac genes in LDN and COM-1 iPSC-CM
A; Images showing LDN-1, LDN-2, and COM-1 iPSC-CM after 7 days in culture. *B;* qPCR data comparing the mRNA expression in LDN-1, LDN-2, and COM-1 cells. Genes quantified were actinin-2 (ACTN2) as well as various cardiac ion channels: Cav1.2 (CACNA1C), hERG (KCNH2), Kir2.1(KCNJ2), Nav1.5 (SCN5A), and the ryanodine receptor (RYR2).

2. Comparison of impedance for LDN-1 and COM-1

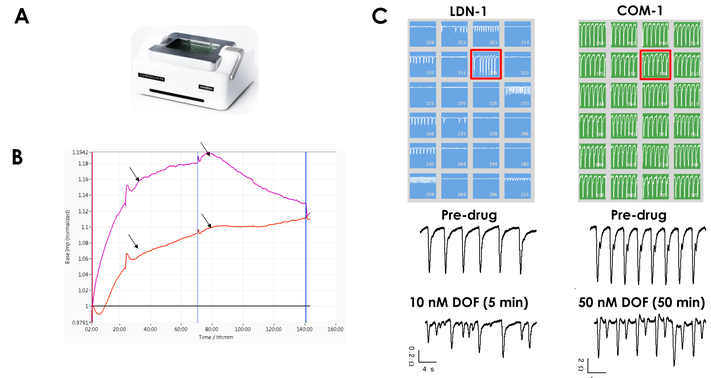
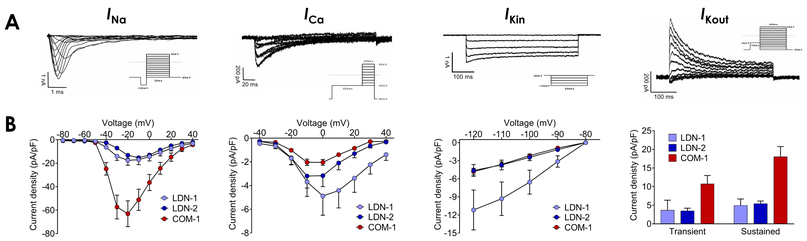


Figure 3: Comparison of spontaneous impedance parameters from LDN-1 and COM-1 iPSC-CM
A; The CardioExcyte 96 platform (Nanion Technologies) combines impedance and EFP to measure effects on contractility and ion channel modulation. *B;* Development of the base impedance over 6 days for LDN-1 cells (normalised to 2 h time point). The wells with beating cells (n = 18) showed a higher base impedance (pink) than the wells without beating cells (n = 24; red). Arrows indicate artefacts caused by medium exchange at 24 and 72 h. *C;* Top panels show snapshot of control impedance traces. LDN-1 cells showed poor well-to-well reproducibility compared to COM-1. Bottom panels show dofetilide triggered arrhythmias in both iPSC-CM (data from the wells highlighted in red).

3. Expression of key ionic currents in LDN iPSC-CM



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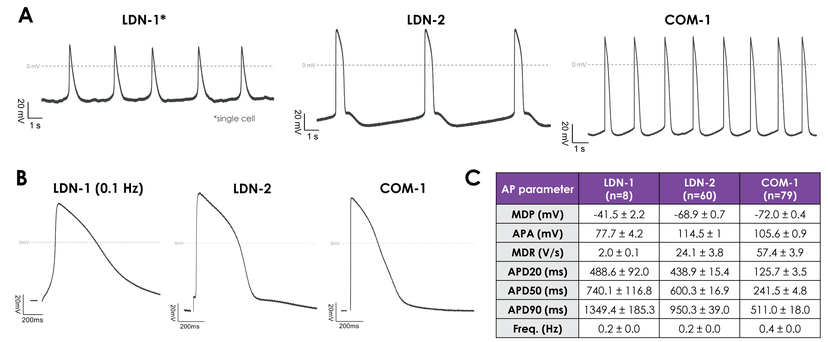
Cell line	I_{Na} (pA.pF^{-1})	I_{Ca} (pA.pF^{-1})	Ratio of I_{Na}:I_{Ca}	I_K expression (number of cells)		
				Inward	I_{Kout} transient	I_{Kout} sustained
LDN-1	-17.6 ± 4.2	-4.9 ± 1.6	4:1	14/14	8/12	12/12
LDN-2	-15.3 ± 2.5	-3.2 ± 1.0	5:1	6/6	3/15	5/15
COM-1	-67.9 ± 12.4	-2.0 ± 0.3	34:1	5/7	16/18	14/18

Figure 4: Functional expression of cardiac ionic currents in LDN and COM-1 iPSC-CM
LDN cells express higher levels of L-type calcium ($I_{Ca,L}$), and inward I_K (I_{Kin}) currents compared to COM-1. Sodium (I_{Na}) and outward I_K (I_{Kout}) current expression was significantly lower in LDN cells. *A;* Representative currents from LDN-1 cells for I_{Na}, $I_{Ca,L}$, I_{Kin} and I_{Kout} elicited by the voltage protocols shown. *B; I-V* relationships for I_{Na} (peak), $I_{Ca,L}$ (peak), and I_{Kin} (end of the pulse), and the mean current densities for transient I_{Kout} (peak) and sustained I_{Kout} (end of the pulse). *C;* Table summarising the mean current densities, I_{Na}:I_{Ca} ratio and I_K expression in all three cell types. Data presented as mean ± SEM.

3. Characterisation of spontaneous and evoked AP



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AP parameter	LDN-1 (n=8)	LDN-2 (n=60)	COM-1 (n=79)
MDP (mV)	-41.5 ± 2.2	-68.9 ± 0.7	-72.0 ± 0.4
APA (mV)	77.7 ± 4.2	114.5 ± 1	105.6 ± 0.9
MDR (V/s)	2.0 ± 0.1	24.1 ± 3.8	57.4 ± 3.9
APD20 (ms)	488.6 ± 92.0	438.9 ± 15.4	125.7 ± 3.5
APD50 (ms)	740.1 ± 116.8	600.3 ± 16.9	241.5 ± 4.8
APD90 (ms)	1349.4 ± 185.3	950.3 ± 39.0	511.0 ± 18.0
Freq. (Hz)	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0

Figure 5: LDN and COM-1 recordings of spontaneous and evoked action potentials in control conditions
Representative spontaneous (A) and evoked (B) AP traces from LDN-1, LDN-2, and COM-1 cells. AP profiling showed variation of intrinsic electrical activity of the different cell lines. LDN-1 cells had a depolarised MDP, produced very small AP and could not be paced at 1 Hz which limited their usability for further pharmacological characterisation. LDN-2 cells had a resting membrane potential and AP properties that were more comparable to COM-1 cells. *C;* Table summarising the average spontaneous AP parameters for each cell type in control conditions. Data presented as mean ± SEM.

4. Core Cardiac Channel Pharmacology

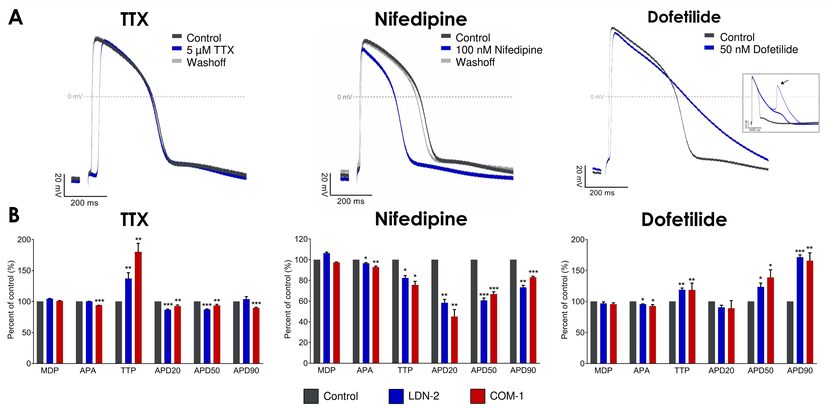

Figure 6: LDN-2 and COM-1 iPSC-CM display comparable responses to core cardiac channel inhibitors
A; Representative traces from LDN-2 cells (1 Hz) under control conditions (grey) and in the presence of 5 µM TTX, 100 nM nifedipine or 50 nM dofetilide (inset shows EAD generation at 0.1 Hz). *B;* Average effect (% of control) for each compound on AP parameters for LDN-2 (blue) and COM-1 (red) cells. Data presented as mean ± SEM, n ≥ 4. Significance calculated by a paired two-tailed Student's *t*-test, * p<0.05, ** p<0.01, *** p<0.001.

Conclusions

- Both LDN-1 and LDN-2 cells lines tended to express higher levels of cardiac markers and ion channel mRNA compared to COM-1.
- Impedance measurements showed that LDN-1 cells are beating after 2-3 days in culture, and elicit arrhythmic beating in response to dofetilide application.
- Electrophysiological characterisation of LDN iPSC-CM showed both cell lines express the three major ionic currents (I_{Na}, $I_{Ca,L}$, and I_K) and are able to generate spontaneous and evoked AP.
- LDN-2 cells had more consistent AP activity and their core channel pharmacology was largely comparable to COM-1 cells (including EAD generation with I_{Kr} inhibitors).
- AP differences between LDN-2 and COM-1 cell lines may be due in part to low functional expression of I_{Na} in LDN-2 iPSC-CM.
- Whilst LDN-2 cells exhibit the expected core cardiac AP pharmacology, further development is required to emulate the key functional properties of commercial iPSC-CM products.

Acknowledgements

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