Na\textsubscript{V}1.5 knockout in iPSCs: a novel approach to study Na\textsubscript{V}1.5 variants in a human cardiomyocyte environment

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Cardiomyocytes derived from patient-specific induced pluripotent stem cells (iPSC-CMs) successfully reproduce the mechanisms of several channelopathies. However, this approach involves cell reprogramming from somatic tissue biopsies or genomic editing in healthy iPSCs for every mutation found and to be investigated. We aim to knockout (KO) Na\textsubscript{V}1.5, the cardiac sodium channel, in a healthy human iPSC line, characterize the model and then, use it to express variants of Na\textsubscript{V}1.5. We develop a homozygous Na\textsubscript{V}1.5 KO iPSC line able to differentiate into cardiomyocytes with CRISPR/Cas9 tool. The Na\textsubscript{V}1.5 KO iPSC-CMs exhibited an organized contractile apparatus, spontaneous contractile activity, and electrophysiological recordings confirmed the major reduction in total Na\textsuperscript{+} currents. The action potentials (APs) exhibited a reduction in their amplitude and in their maximal rate of rise. Voltage optical mapping recordings revealed that the conduction velocity Ca\textsuperscript{2+} transient waves propagation velocities were slow. A wild-type (WT) Na\textsubscript{V}1.5 channel expressed by transient transfection in the KO iPSC-CMs restored Na\textsuperscript{+} channel expression and AP properties. The expression of Na\textsubscript{V}1.5/delQKP, a long QT type 3 (LQT3) variant, in the Na\textsubscript{V}1.5 KO iPSC-CMs showed that dysfunctional Na\textsuperscript{+} channels exhibited a persistent Na\textsuperscript{+} current and caused prolonged AP duration that led to arrhythmic events, characteristics of LQT3.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AP           | Action potential |
| APD          | Action potential duration |
| CRISPR/Cas9  | Clustered regularly interspaced short palindromic repeats/associated protein 9 |
| CHO          | Chinese hamster ovary |
| CICR         | Calcium-induced calcium release |
| cTnT         | Cardiac troponin T |
| DAD          | Delayed afterdepolarization |
| DelQKP       | Deletion of three amino acids QKP (glutamine, lysine, proline) |
| dV/dt\textsubscript{max} | Upstroke velocity |
| EAD          | Early postdepolarization |
| HEK          | Human embryonic kidney |
| iPSC-CM      | Cardiomyocytes derived from induced pluripotent stem cells |
| KO           | Knock-out |
| LQT3         | Long QT syndrome type 3 |
| Mlc2v        | Ventricular myosin light chain 2 |
| OM           | Optical mapping |
| SCN5a        | Sodium voltage-gated channel alpha subunit 5 |
| TDP          | Torsade de pointes |
| TTX          | Tetrodotoxin |
| VGSC         | Voltage-gated sodium channel |
| WT           | Wild type |

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The human SCN5A gene located on chromosome 3p21 encodes the voltage-gated sodium channel (VGSC) α subunit, the predominant Na⁺ channel in the heart. Na⁺,1.5 plays a vital role in triggering and shaping cardiac action potentials (APs). Its biophysical properties contribute to controlling phase zero of APs and their durations (APDs), and define the speed of propagation (conduction velocity) within the heart. Over the past 25 years, numerous SCN5A mutations have been associated with arrhythmic disorders, including congenital long QT syndrome type 3 (LQT3), Brugada syndrome (BrS), atrial fibrillation (AFib), progressive cardiac conduction defect (PCCD), sinus node dysfunction (SND), sudden infant death syndrome (SIDS), and dilated cardiomyopathy.

Most of the functional studies on specific SCN5A mutations have relied on heterologous expression systems. The first studies were performed in vitro using Xenopus oocytes. Subsequently, cell lines such as human embryonic kidney (HEK293) cells and Chinese hamster ovary (CHO) cells were used. They provided a platform for characterizing the biophysical properties of Na⁺ channels and their variants. These heterologous expression systems remain in wide use today. The biophysical parameters of mutated Na⁺ channels obtained from electrophysiological recordings in these expression systems have been extrapolated to deduce their effect on cardiac function. The use of computational simulations to explore how Na⁺,1.5 variants affect cardiac excitability by simulating AP firings has also proven useful for understanding their clinical impact. However, these expression and computational models have limitations. Although the Na⁺,1.5 α subunit acts as a full-fledged Na⁺ channel in vivo, it is part of a large multiprotein complex. Cytoskeletal, regulatory, cell adhesion, trafficking, and gap junction proteins have been found in close association with Na⁺,1.5, several of which alter the biological and/or biophysical properties of the channel.

Mouse models emerged as a tool for in vivo studies and a way to bypass the limitations of heterologous systems. The generation of transgenic mice carrying knockout channels or specific ion channel mutations has opened the way to studying the underlying disease mechanisms. However, mouse models are limited by the fundamental differences in cardiac electrophysiology between mice and humans and the lack of general applicability of the results to human diseases. In addition, the generation of such models is expensive, laborious, and far less straightforward than heterologous systems for studying and screening the functional effects of Na⁺,1.5 variants.

Ideally, mutations associated with cardiac arrhythmias should be studied in the native cardiomyocyte environment. However, obtaining ventricular cardiac biopsies is a highly invasive procedure and is not without significant risk to the patient. Recent advances in induced pluripotent stem cells (iPSCs) have made it possible to generate an unlimited number of human cardiomyocytes (iPSC-CMs) in vitro from healthy individuals and from patients with cardiac abnormalities using specific differentiation protocols. These advances have made it possible, among other things, to study the molecular determinants of channelopathies caused by mutated Na⁺,1.5 channels in a human cardiac context. When somatic tissues are unavailable, genomic editing with CRISPR/Cas9 tools, for example, can be used to introduce the mutation to be characterized into a control iPSC line and to then differentiate it into cardiomyocytes. Once again, this process of establishing an iPSC line for each Na⁺,1.5 variant of interest is a laborious process and lacks the throughput of heterologous expression systems.

In the present study, we established an iPSC model that can be used to easily study various Na⁺,1.5 channels variants in a human cardiac background. Our model is based on an iPSC line where the CRISPR/Cas9 genomic editing tool was used to permanently knock-out (KO) Na⁺,1.5. We successfully differentiated this Na⁺,1.5 KO cell line into cardiomyocytes and characterized the impact of the KO on cardiac function. We then used these cardiomyocytes (Na⁺,1.5 KO iPSC-CMs) to express, by simple transfection, a mutated Na⁺,1.5 channel (Na⁺,1.5/delQKP) associated with LQT3 and to compare it with wild-type channel-transfected cells (Na⁺,1.5/WT). The present study is a proof-of-concept for the use of Na⁺,1.5 KO iPSC-CMs as an expression system to further characterize the impact of Na⁺,1.5 variants in a human cardiac background.

**Methods**

**iPSC cultures and cardiomyocyte differentiation.** All the work with hiPSCs were approved by CIUSSS de la Capitale-Nationale ethic committee (Project #2019-1734). Human iPSCs were grown on hESC-qualified Matrigel (Corning, NY, USA) in mTeSRplus medium (StemCell Technologies, BC, Canada) and were routinely passaged with ReLeSR (StemCell Technologies). The iPSCs were produced at the LOEX core facility (Quebec, QC, Canada) as described previously. The iPSCs were differentiated into cardiomyocytes (CMs) with a monolayer-based protocol using STEMdiff™ Cardiomyocyte differentiation kits (StemCell Technologies) according to the manufacturer's protocol and instructions. The differentiation process was very efficient, and spontaneously beating cells were observed at day 8 to 12 of differentiation. The iPSC-CM were maintained in STEMdiff™ Cardiomyocyte Maintenance media (StemCell Technologies) until the day of experiment.

**Generation of the NaV1.5 KO human iPSC line.** The sgRNAs for CRISPR editing were designed using Predesigned Alt-R CRISPR-Cas9 guide RNA tool (Integrated DNA technologies website) and chosen according to the on-target and off-target scores. We have selected the following sgRNAs which have higher scores and the fewest and the non-cardiac off-targets: sgRNA-1 (5′-GATTTCACCCCTTCAATCAT) and sgRNA-2 (5′-ACT TTAGCTGAGATCCTGAG). The potential off-target sites are listed in the Supplementary Table S1 online.

The sgRNAs were cloned into the same modified px458 plasmid (Addgene plasmid 48,138) coding for the S. aureus Cas 9. Each sgRNA was under the control of its own U6 promoter. To generate CRISPR KO iPSCs, 7.5 μg of knockout plasmid (px458-sgRNA1-sgRNA2) was nucleofected into 800,000 iPSCs with 4D-Nucleofector (Lonza, Bäle, Switzerland) using P3 Primary cell kits (Lonza) and program CA-137. The cells were then seeded in 35-mm dishes in multiple dilutions in mTeSRPlus supplemented with clone R (StemCell Technologies). After ~7 days in culture, each iPSC colony was manually picked using a micro-pipette under a Lynx EVO scope (Vision Engineering, CA, USA), and was transferred into two individual wells of two 96-well plates containing mTeSRPlus medium. One plate used to extract the genomic DNA for PCR screening to detect positive clones, and
the other was used to maintain and expand the positive clones. The PCR screening used the F1, 5′-GACCAGAAA TGCACCTTGCTCTCTGTA and R1, 5′-GGTGTTGCTGAGATTGAGGTGCCGCTTTGCT primers, which target regions surrounding the cutting sites of the sgRNAs. Positive clones, those with exon 6a removed, are characterized by a 682-bp PCR amplicon while negative clones are characterized by a 962-bp amplicon (Fig. 1B). One positive clone was chosen for the present study and was named NaV1.5 KO.

cDNA analysis. RNA was extracted from NaV1.5 KO iPSC-CMs and the control cell line on day 30 of maturation using Quick RNA mini-prep kits (Zymo Research, CA, USA). cDNA was synthesized using the Proto-
They were resolved on 4–12% gradient stain-free SDS–polyacrylamide gels (Bio-Rad) and were blotted on 0.45-
4°C. Protein concentrations were measured using the DC protein assay (BioRad, ON, Canada), with BSA as a reference. Protein extracts (20 μg) were denatured in 2X Laemmlı buffer (MilliporeSigma) at 37 °C for 30 min. They were resolved on 4–12% gradient stain-free SDS–polyacrylamide gels (Bio-Rad) and were blotted on 0.45-

Western blotting. The proteins of the iPSC-CMs were extracted on day 30 of maturation. Briefly, the cells were washed with PBS and were lysed by scraping the cells into lysis buffer (10 mM Tris, 1% Nonidet P-40, 0.5% deoxycholic acid) supplemented with protease inhibitor cocktail (MilliporeSigma, ON, Canada). The cells were incubated in the lysis buffer for 1 h at 4 °C. Then, they were clarified by centrifugation at 18,000 g for 15 min at 4 °C. Protein concentrations were measured using the DC protein assay (BioRad, ON, Canada), with BSA as a reference. Protein extracts (20 μg) were denatured in 2X Laemmlı buffer (MilliporeSigma) at 37 °C for 30 min. They were resolved on 4–12% gradient stain-free SDS–polyacrylamide gels (Bio-Rad) and were blotted on 0.45-

Immunofluorescence staining. Immunofluorescence staining of iPSC-CMs was carried out on day 30 of maturation. The cells were dissociated from the monolayers using STEMDiff™ Cardiomyocyte Dissociation kits (StemCell Technologies) and were plated on Matrigel-treated 13-mm TC Coverslips (Sarstedt, QC, Canada). The cells were fixed in a mixture of 4% paraformaldehyde and 4% sucrose in PBS for 10 min. Then, they were washed and were permeabilized for 30 min at room temperature in a mixture of 0.1% Triton X-100, 1% BSA, and 5% goat serum in PBS. The cells were incubated overnight at 4 °C with the following antibodies: rabbit anti-MLC2v (1/300, Cat #A11005 and Cat #A11008, ThermoFisher Scientific, USA). DAPI was used to counterstain the nuclei. The cells were observed using a Zeiss LSM confocal microscope.

iPSC-CM transfection. The iPSC-CMs were transfected at days 25–30 of maturation using Viafect™ Transfection Reagent according to an online protocol (Cat #E4981, Promega Corporation, WI, USA). The cells were transfected with the pCDNA3.1 vector containing either WT or delQKP NaV1.5 cDNA (500 ng) and with the pmGFP-N1 (250 ng) vector to select transfected cells based on green fluorescence signal. The medium was changed after 24 h, and the iPSC-CMs were dissociated into single cells in 35-mm dishes using STEMDiff™ Cardiomyocyte Dissociation kit (StemCell Technologies). Patch-clamp experiments were performed 3–7 days post-dissociation.

Electrophysiology. Patch clamp experiments with the iPSC-CMs were performed at room temperature using an Axopatch 200B amplifier and pClamp software v10 (Molecular Devices, CA, USA). Macroscopic Na+ currents and APs were recorded using the whole-cell configuration of the patch clamp technique in voltage- and current-clamp modes, respectively. The pipettes were made from borosilicate glass capillaries (Sutter Instrument, CA, USA) and were fire polished.

For the voltage-clamp experiments, the pipettes were coated with HIPEC (Dow-Corning, MI, USA) to minimize electrode capacitance. The pipettes were filled with a solution containing (in mmol/L): 35 NaCl, 105 CsF, 10 EGTA, and 10 HEPES. The pH was adjusted to 7.4 with CsOH. To record biophysical parameters, the bath solution was composed of (in mmol/L): 154 NaCl, 5.6 KCl, 2 CaCl2, 1 MgCl2, 10 D-glucose, 10 HEPES, 10 TEA-Cl, and 0.01 Nifedipine. For persistent Na+ currents recordings, the bath solution was replaced by (in mmol/L): 105 NaCl, 2 KCl, 1.5 CaCl2, 1 MgCl2, 10 D-glucose, 10 HEPES, 10 TEA-Cl, 0.01 nifedipine, and 20 μmol/L TTX (LATOXAN, France). The pH of the bath solutions was adjusted to 7.4 with methanethiosulfonic (MTS) acid. Series resistance and cell capacitance were corrected. Na+ currents were filtered at 5 kHz, digitized at 10 kHz, and stored on a microcomputer equipped with an AD converter (Digidata 1440A, Molecular Devices). P/4 leak subtraction was used prior to applying pulse stimulations.

For the current-clamp experiments, the patch pipettes (resistance 2–5 mΩ) were filled with a solution containing (in mmol/L): 10 NaCl, 122 KCl, 1 MgCl2, 1 EGTA, and 10 HEPES. The pH was adjusted to 7.3 with KOH. The bath solution (external current clamp) was composed of (in mmol/L): 154 NaCl, 5.6 KCl, 2 CaCl2, 1 MgCl2, 8 D-glucose, and 10 HEPES. The pH was adjusted to 7.3 with NaOH. The APs were recorded at 0.5, 1, 2 and 2.5 Hz stimulation frequencies. The holding potential during recording was maintained at −80 mV. The duration of stimulation pulse was 3 ms with 0.5–1.5 nA injected current depending on the cell.

Optical mapping of iPSC-CMs in monolayers. Optical mapping techniques were used to study the membrane potential and calcium waves on iPSC-CMs monolayers. The iPSC-CMs monolayers were reconstituted with the same number of cells over the experiments. iPSC-CMs were dissociated at days 12 to 15 of maturation with STEMDiff™ Cardiomyocyte Dissociation kit (StemCell Technologies). The monolayer was then reconstituted with 350,000 cells seeded on a 13 mm TC coverslip (Sarstedt) coated with hESC-qualified Matrigel®. The STEMDiff™ Cardiomyocyte Maintenance medium was replaced every two days for at least 15 days post-reconstruction. The iPSC-CMs (30–60 days of maturation) were fed with fresh medium one hour before
NaV antibody which recognize the intracellular loop between domains III and IV, identical in all isoforms of the same copy number that control except for the X chromosome where it was divided by two. The NaV1.5 KO cell was compared to a genomic DNA control provided in the hPSC Genetic Analysis Kit. The NaV1.5 KO iPSCs has the chromosomes status (Supplementary Fig. S1 online). 8 most mutated regions in iPSCs were analysed and

in mmol/L: 154 NaCl, 5.6 KCl, 2 CaCl2, 1 MgCl2, 8 D-glucose, and 10 HEPES; pH 7.3). The cells were washed with the Tyrode solution and incubated for an additional 10 min or 30 min before optical or Ca2+ imaging, respectively. Membrane voltage and intracellular Ca2+ transients were mapped at high-speed (500 frames/sec) using CMOS N256 camera (MiCAM03, Brainvision, SciMedia Ltd, U.S.A) at a field of view of 15 mm. The imaging system includes a 530 nm green LED light source (LEX2-LZ4-G) with a stable intensity of 360 mW/cm2, led by an optical fiber and followed by a imaging container comprising a collagenator, a dichroic mirror (560 nm), a band-pass 531/40 nm excitation filter (50 mm, BrightLine®, Semrock) and a long-pass 665 nm emission filter (50 mm, Andover Corporation), as well as a lens system with a maximum aperture of f/1.4 aligned in epifluorescence configuration for di-4-ANEPPS and Rhod-2, AM imaging. The recordings were performed at 37 °C using TCW1 worming plate controlled by TC02 temperature controller (Multichannel Systems, BW, Germany). Bipolar platinum/iridium electrodes (World Precision Instruments) positioned at the lower edge of the preparation were used to pace the monolayer with a stimulus generator (STG4002, Multichannel Systems) at cycle of 500 ms to 1500 ms using a bipolar square pulse of ± 8 V of a duration of 10 ms. 10 µmol/L of blebbistatin (Sigma-Aldrich) was added to the recording solution during membrane voltage recording to prevent cardiomyocytes contraction artefacts in the fluorescence signal. Raw optical signals were processed using Brainvision Workbench software to analyse the electrophysiological measurements, calculate the conduction velocities and generate the maps.

**Statistical analysis.** In patch clamp recordings, APs exhibited diverse shapes that can be attributed to different cardiomyocyte cell types, that are, ventricular, atrial, and nodal cells. For statistical analyses, we decided to pool ventricular and atrial-like APs and exclude nodal-like APs. The nodal-like APs were excluded based on their very distinctive shape, i.e., no plateau and low overshoot.

All statistical analyses were performed using PRISM8 software (GraphPad, CA, USA). The normality of distribution was determined using D’Agostino-Pearson normality test. When the distribution is normal, the data are expressed as mean ± SEM (standard error of the mean). If not, the data are expressed as median ± quartiles (25 % and 75 %) with the min to max values. When 3 groups were compared, statistical significance was determined by one-way ANOVA with Dunnet’s post hoc test, or a two-way ANOVA for two independent variables. When only two groups were compared, a two-tailed unpaired Student’s t-test was used. Where data were not normally distributed, a nonparametric test (Mann–Whitney or Kruskal–Wallis test) was performed. All the statistical tests were performed using a 95 % confidence interval and the differences were considered significant beyond the risk threshold 0.05% (*P < 0.05, **P < 0.01, ***P < 0.001).

**Results**

**CRISPR-Cas9 strategy for NaV1.5 knockout.** To selectively and permanently suppress NaV1.5 channel expression in iPSCs, we used CRISPR-Cas9 gene editing technology. The strategy was to remove exon 6a in the SCN5a gene using two sgRNAs that precisely target the regions flanking this exon (Fig. 1A). We used PCR amplifications of the SCN5a genomic locus encompassing the sgRNA target sites to screen for positive iPSC clones. Figure 1B shows the positive PCR result of the NaV1.5 KO cell line used in the present study. The NaV1.5 KO cells had a shorter amplicon than the control cells (not exposed to Cas9), confirming that exon 6a in SCN5a had been deleted. A genetic screening by quantitative PCR was performed of the NaV1.5 KO iPSCs to verify the chromosomes status (Supplementary Fig. S1 online). 8 most mutated regions in iPSCs were analysed and compared to a genomic DNA control provided in the hPSC Genetic Analysis Kit. The NaV1.5 KO iPSCs has the same copy number that control except for the X chromosome where it was divided by two. The NaV1.5 KO cell line comes from a female, containing one copy of X chromosome, while the genomic DNA control comes from a female. So, the KO line does not appear to have genetic abnormalities.

To verify that NaV1.5 protein expression was suppressed in the cells, the NaV1.5 KO iPSCs were differentiated into cardiomyocytes. After 30 days of maturation, RNA and Western blot analyses were performed. The NaV1.5 cDNA PCR amplification revealed a shorter amplicon, confirming that the NaV1.5 mRNA was free of exon 6a and 6b as shown by the sequencing chromatogram (Fig. 1C,D). The deletion of the two exons 6 in the mRNA instead of only one as designed by our strategy can probably be explained by a mis-splicing event. The deletion of the exon 6a may have compromised splicing sites for the exon 6b since they are only 140 bp apart in the SCN5a gene. The NaV1.5 cDNA sequencing chromatogram validated the shift in the reading frame of NaV1.5 and the formation of stop codons in the region corresponding to exon 7 (only the first stop codon is shown in Fig. 1D). The Western blot analysis were performed using two primary antibodies (Fig. 1E). The anti-NaV1.5 antibody which recognize the intracellular loop of NaV1.5 channel, between domains I and II and the anti-Pan NaV antibody which recognize the intracellular loop between domains III and IV, identical in all isoforms of NaV1 in all vertebrates. The unedited blots are available at different exposures in Supplementary Figure S6 and S7. The immunostaining shows that NaV1.5 protein expression was completely suppressed in the KO iPSC-CMs. Our CRISPR construction makes it possible to stop the translation of the protein at the end of domain I, that is why we cannot observe a truncated NaV1.5 protein in the KO iPSC-CMs. The immunostaining with an anti-Pan NaV antibody that recognizes all channel subtypes showed that isoforms other than NaV1.5 were expressed but at much lower levels (Fig. 1E). We used this new NaV1.5 KO iPSC line in the present study.

**Intact contractile structures in NaV1.5 KO iPSC-CMs.** The NaV1.5 KO iPSCs were successfully differentiated into cardiomyocytes (Supplementary Videos S1 and S2 online). No difference in the differentiation course of the KO cells was observed compared to the control cells. The NaV1.5 KO iPSC-CMs started to sponta-
neously beat around day 8 to 10 of differentiation as did the control cells. After 30 days of maturation, the beating rate of the Na\textsubscript{v}1.5 KO iPSC-CMs (0.46 ± 0.30 Hz) was also similar to that of the control cells (0.46 ± 0.19 Hz) (Fig. 5I). The morphologies of the cardiomyocytes differentiated from the Na\textsubscript{v}1.5 KO iPSC line were then characterized to rule out the possibility that the loss of Na\textsubscript{v}1.5 had affected sarcomeric organization. Immunofluorescence staining for myosin light chain 2v (MLC2v, green staining) and cardiac troponin T (cTnT, red staining). The bottom panels show merged images. Scale bar: 20 µm. Images were acquired using a Zeiss ImagesM2 LSM confocal microscope and were processed using ZEN software (Zeiss). This figure was made using IMAGEJ 1.52i (Java 1.8.0_66, http://imagej.nih.gov/ij).

Voltage and current-clamp analyses of Na\textsubscript{v}1.5 KO iPSC-CMs. The Na\textsubscript{v}1.5 channel is by far the main contributor to Na\textsuperscript{+} currents that initiate and trigger the rise of APs in cardiomyocytes. With the KO of Na\textsubscript{v}1.5, we expected that the iPSC-CMs would produce a very small Na\textsuperscript{+} current. We thus performed voltage-clamp recordings in dissociated iPSC-CMs cells to measure the Na\textsuperscript{+} and Ca\textsuperscript{2+} currents in the Na\textsubscript{v}1.5 KO and control cells. The Na\textsubscript{v}1.5 KO iPSC-CMs did not produce any recordable Na\textsuperscript{+} current, whereas the control cells generated a mean current amplitude of −3200 pA, as shown by the representative current traces (Fig. 3A). When
the Na⁺ concentration was increased from 35 to 140 mmol/L in the external recording solution, the Naᵥ1.5 KO iPSC-CMs produced a small Na⁺ current of –670 pA, as shown by the representative current trace (Fig. 3A). At this Na⁺ concentration, the amplitudes of the Na⁺ currents in the control cells were too large (> −20,000 pA) to be recorded. Given that the Naᵥ1.5 KO cell line generated beating cardiomyocytes, it should also produce Ca²⁺ currents that are responsible for excitation–contraction coupling in cardiac muscles. The Naᵥ1.5 KO produce a Ca²⁺ current which has the same biophysical properties that control iPSC-CMs (Supplementary Fig. S2 online).
In the Na\textsubscript{v},1.5 KO iPSC-CMs, the small residual Na\textsuperscript{+} current was blocked by 50 nmol/L TTX (Supplementary Fig. S3 online). The Ca\textsuperscript{2+} current activated several milliseconds after the Na\textsuperscript{+} current was blocked with 5 µmol/L nifedipine, a Ca\textsuperscript{2+} channel blocker.

We next studied the impact of Na\textsubscript{v},1.5 current losses on the properties of APs in iPSC-CMs. Na\textsubscript{v},1.5 KO and control iPSC-CMs both triggered spontaneous APs in the gap-free mode of the current-clamp configuration (data not shown). We observed similar cell-subtypes repartition between control and Na\textsubscript{v},1.5 KO iPSC-CMs: most ventricular-like cells and a minority of atrial- and nodal-like cells (data not shown). When stimulated at a stimulation frequency of 1 Hz, the Na\textsubscript{v},1.5 KO cells triggered AP trains that respected the pace of stimulation seen in the control cells (Fig. 3B). However, the injected current to trigger an AP in the Na\textsubscript{v},1.5 KO cells (0.97 ± 0.05 nA) was significantly increased compared to control cells (0.77 ± 0.04 nA; **p < 0.01 unpaired t-test). The following experiments were carried out on cardiomyocytes stimulated at 1 Hz in order to normalize the beating rate and enable further analysis. For each AP, several parameters were analysed, this include the overshoot, the dV/dt\textsubscript{max} which corresponds to the opening of Na\textsuperscript{+} channels (0 phase of AP), and the 20, 50 and 90% of repolarization which corresponds to the opening of Ca\textsuperscript{2+} and K\textsuperscript{+} channels (1, 2 and 3 phases of AP).

As expected, several alterations in the APs from Na\textsubscript{v},1.5 KO cells were identified. The APs from the Na\textsubscript{v},1.5 KO iPSC-CMs exhibited a significant increase in duration (APD) when measured at 20, 50, and 90% of repolarization compared to the control cells (Fig. 3C,D). For example, the median of APD\textsubscript{90} was 442.5 ms with quartiles [333.9; 522.0] for the Na\textsubscript{v},1.5 KO cells compared to 319.3 ms [200.3; 452.8] for the control cells, which was a significant increase of 122 ms. The AP overshoot and the upstroke velocity (dV/dt\textsubscript{max}) were also affected in the Na\textsubscript{v},1.5 KO iPSC-CMs, which influenced the amplitude and the time to peak of APs, as shown in the representative traces (Fig. 3E). The overshoot in the Na\textsubscript{v},1.5 KO iPSC-CMs (37.0 ± 2.2 mV) decreased significantly by 14 mV compared to the control cells (51.4 ± 2.5 mV) (Fig. 3F). The main effect on APs was related to the dV/dt\textsubscript{max} where a major decrease of 88 mV/ms was observed in the Na\textsubscript{v},1.5 KO iPSC-CMs (15.2 ± 1.4 mV/ms) compared to the control cells (102.8 ± 7.7 mV/ms) (Fig. 3G).

From a pharmacological point of view, Na\textsubscript{v},1.5 KO does not respond in the same way as the control iPSC-CMs. The APs from control iPSC-CMs were sensitive to TTX treatment (Supplementary Fig. S4 online). Indeed, the addition of TTX decreased the dV/dt\textsubscript{max} of APs from control cells and this in a concentration-dependent way. For Na\textsubscript{v},1.5 KO iPSC-CMs, the TTX had no blocking effect of the triggering of APs (Supplementary Fig. S3 online). Moreover, the treatment with 1 µmol/L nifedipine blocks the APs from the Na\textsubscript{v},1.5 KO but not APs from the control iPSC-CMs which just had a shortened duration (Supplementary Fig. S3 and S5 online). These results confirm the Ca\textsuperscript{2+} nature of the APs recorded from Na\textsubscript{v},1.5 KO iPSC-CMs.

Voltage and calcium optical mapping of iPSC-CM monolayers. We next generated large (~1 cm diameter) iPSC-CM monolayers to study the biophysical impact of the loss of Na\textsubscript{v},1.5 channels at level closer to heart tissue using the optical mapping (OM) technique. The conduction of electrical impulses through the iPSC-CM monolayers stained with the voltage sensitive dye di-4-ANEPPS was first analyzed. Figure 4A shows representative optical APs (OAPs) recorded in Na\textsubscript{v},1.5 KO and control monolayers stimulated at 1 Hz. As with the patch-clamp recordings, both cell lines generated AP trains that followed the pace of the electrical stimulations. Activation maps were produced for the Na\textsubscript{v},1.5 KO and control monolayers from these optical recordings (Fig. 4B). The maps notably showed that the conduction velocity (CV) was slower in the Na\textsubscript{v},1.5 KO monolayers than in the control monolayers (Supplementary Video S3 online). When stimulated at 0.5, 1, and 1.5 Hz, the conduction velocities measured in the Na\textsubscript{v},1.5 KO monolayers were 2.67 ± 0.16, 2.43 ± 0.13, and 2.12 ± 0.13 cm/s, respectively compared to 13.02 ± 1.7, 10.49 ± 1.3, and 8.77 ± 1.4 cm/s in the control monolayers, respectively (Fig. 4C). We next analyzed the parameters of the OAPs and found that the rise time, the duration, and the amplitude were affected in the Na\textsubscript{v},1.5 KO monolayers at all three stimulation frequencies (Fig. 4D). The rise time of the OAPs was prolonged in the Na\textsubscript{v},1.5 KO monolayers by an average of 5.7 ms. For example, the rise time was 16.5 ± 0.6 and 10.7 ± 1.0 ms for the Na\textsubscript{v},1.5 KO and the control monolayers at 1 Hz, respectively. The duration of the OAPs was significantly shortened for the Na\textsubscript{v},1.5 KO monolayers (Fig. 4E; Supplementary Video S3 online). At 1 Hz, the APD\textsubscript{50} and APD\textsubscript{80} were 356 ± 15 and 421 ± 14 ms for the Na\textsubscript{v},1.5 KO monolayers, and 489 ± 24 and 585 ± 22 ms for the control monolayers. This represented 133 ms and 164 ms differences in the APD\textsubscript{50} and APD\textsubscript{80} respectively. The amplitude of the OAPs also significantly decreased by 9 %, as calculated using the normalized fluorescence intensity, decreasing from 59,900 relative fluorescence units (RFU) in the control monolayers to 54,275 RFU in the Na\textsubscript{v},1.5 KO monolayers at 1 Hz (Fig. 4G).

The spatial changes in the kinetics and amplitude of Ca\textsuperscript{2+} transients were next studied using the fluorescent probe Rhod-2-AM on cell monolayers. Figure 5A shows representative Ca\textsuperscript{2+} signal recorded in Na\textsubscript{v},1.5 KO and control monolayers when electrically stimulated at 1 Hz. Uniform propagation of Ca\textsuperscript{2+} waves through the entire monolayer was observed in both cell lines, as shown by the representative Ca\textsuperscript{2+} activation maps in Fig. 5B. As observed in OAPs recording, Na\textsubscript{v},1.5 KO monolayers exhibited a significantly delayed and slower propagation of Ca\textsuperscript{2+} transients and a compared to control monolayers (Supplementary Video S4 online). This was expected since the Ca\textsuperscript{2+} transients are triggered during AP propagation through the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) process. Thus, Ca\textsuperscript{2+} waves propagation velocities (CaVP) were measured in Na\textsubscript{v},1.5 KO (3.3 ± 0.6, 3.2 ± 0.4 and 2.7 ± 0.5 cm/s) and control (13.4 ± 1.5, 10.3 ± 0.95 and 7.4 ± 0.9 cm/s) at 0.5, 1 and 1.5 Hz pacing frequencies, respectively (Fig. 5C). Similarly, the Ca\textsuperscript{2+} transient characteristics in Na\textsubscript{v},1.5 KO monolayers were altered including its amplitude, durations (TD\textsubscript{50,80}), half times to peak and decay speed (τ). A significant reduction of 10% in the Ca\textsuperscript{2+} transients normalized amplitudes was observed between the two groups reflecting the maximum intracellular Ca\textsuperscript{2+} concentrations, as measured at 64,335 RFU in the control and 58,337 RFU in the Na\textsubscript{v},1.5 KO monolayers at stimulation frequency of 1 Hz (Fig. 5D). Furthermore, the durations of Ca\textsuperscript{2+} transients were significantly shortened in Na\textsubscript{v},1.5 KO iPSC-CMs at 0.5 Hz and 1 Hz. For example, TD\textsubscript{50} and TD\textsubscript{80} were reduced by...
85 and 91 ms at 1 Hz, respectively (503 ± 24.4 and 672.8 ± 21.2 ms for NaV1.5 KO, compared to 588.6 ± 23.8 and 763.4 ± 15.9 ms for control) (Fig. 5E,F). More, the half times of Ca²⁺ peak was shortened in NaV1.5 KO iPSC-CMs at 0.5 Hz and 1 Hz, but not at 1.5 Hz (Fig. 5G). At 1 Hz, the half time of Ca²⁺ peak was decreased by 36 ms with 252.3 ± 12.1 and 216 ± 5.8 ms for control and NaV1.5 KO, respectively. As for the decay tau (τ), NaV1.5 KO iPSC-CMs exhibit a faster decay of Ca²⁺ transients (smaller τ) at 0.5 Hz and 1 Hz (Fig. 5H; Supplementary Video S4 online). As an example, the decay τ was 342.6 ± 24.5 in NaV1.5 KO, and 402.5 ± 11.5 in control at 1 Hz. Finally, the spontaneous beating frequencies were measured before the addition of blebbistatin and electrical stimulation for each monolayer. The beating frequencies were found similar at ~0.46 Hz within the two groups (Fig. 5I).
Figure 5. Altered calcium handling in Na\textsubscript{v}1.5 KO iPSC-CMs monolayers. (A) Representative Ca\textsuperscript{2+} transient traces of control and Na\textsubscript{v}1.5 KO iPSC-CMs monolayers. Blue arrows indicate the Ca\textsuperscript{2+} activation delay in Na\textsubscript{v}1.5 KO recording. (B) Representative [Ca\textsuperscript{2+}]\textsubscript{i} Ca\textsuperscript{2+} activation maps at pacing of 1 Hz. The symbol indicating the position of stimulating electrodes, and □ showing the position of the representative recordings. (C) Bar graphs summarizing the Ca\textsuperscript{2+} propagation velocities (CaPVs), (D) the normalized amplitude, (E,F) the Ca\textsuperscript{2+} transient durations at 50 and 80% of the repolarization phase (TD\textsubscript{50} and TD\textsubscript{80}), (G) the half time to peak, and (H) the time constant of repolarization (Decay tau) measured at stimulation frequencies of 0.5, 1 and 1.5 Hz in control (n = 12–15) and Na\textsubscript{v}1.5 KO (n = 6–9). (I) Bar graphs showing the spontaneous Ca\textsuperscript{2+} waves frequencies in control (n = 15) and Na\textsubscript{v}1.5 KO (n = 10) iPSC-CM monolayers. A replicate (n) represents a single monolayer of iPSC-CMs on which the optical mapping was performed. Bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001 as determined by unpaired t-test. This figure was made using MICROSOFT POWERPOINT Version 2106 (build 14131.20278 Office, https://docs.microsoft.com/en-us/) and PRISM 8 Version 8.0.2 (263, https://www.graphpad.com/).
**Na\(_{\alpha, 1.5}\) channel re-expression in Na\(_{\alpha, 1.5}\) KO iPSC-CMs.** We next used the Na\(_{\alpha, 1.5}\) KO cell line as an expression system to characterize the impact of a Na\(_{\alpha, 1.5}\) variant in a human cardiac environment. The known LQT3 variant delQKP\(_{1507-1509}\) (delQKP) was used as a proof-of-concept in the present study. The Na\(_{\alpha, 1.5}\) KO iPSC-CMs were transiently transfected with either the cDNA Na\(_{\alpha, 1.5}\)/delQKP construct or the Na\(_{\alpha, 1.5}\)/WT construct. We conducted a voltage-clamp analysis and compared the biophysical properties of the mutated channel with those of the WT channel. The Na\(_{\alpha, 1.5}\)/WT iPSC-CMs were also compared with the control iPSC-CMs. The Na\(_{\alpha, 1.5}\) KO iPSC-CMs transfected with Na\(_{\alpha, 1.5}\)/WT (Na\(_{\alpha, 1.5}\)/WT) or Na\(_{\alpha, 1.5}\)/delQKP both produced a robust typical Na\(^+\) current (Fig. 6A). The current densities in Na\(_{\alpha, 1.5}\)/WT (− 192 ± 39 pA/pF) and Na\(_{\alpha, 1.5}\)/delQKP (− 195 ± 43 pA) were similar as shown by the I/V curves (Fig. 6B). The Na\(_{\alpha, 1.5}\)/WT tended to have slightly higher current densities compared to control iPSC-CMs (109 ± 10 pA/pF) but the difference was not significant (p = 0.0521) (Fig. 6B). The I/V curves were converted to conductance-voltage curves (G/V), which revealed no significant differences in steady-state activation between Na\(_{\alpha, 1.5}\)/WT and Na\(_{\alpha, 1.5}\)/delQKP (Fig. 6C). Compared to the control iPSC-CMs (− 35.9 ± 1.2 mV), the V\(_{1/2}\) of activation of Na\(_{\alpha, 1.5}\)/WT (− 41.7 ± 1.4 mV) was shifted significantly toward more negative voltages (Fig. 6C). An analysis of steady-state inactivation revealed a significant − 4.6 mV shift in the V\(_{1/2}\) of Na\(_{\alpha, 1.5}\)/delQKP (− 84.8 ± 0.4 mV) compared to Na\(_{\alpha, 1.5}\)/WT (− 80.2 ± 1.1 mV) (Fig. 6D). No difference was seen in the V\(_{1/2}\) of inactivation between Na\(_{\alpha, 1.5}\)/WT and the control iPSC-CMs. The voltage dependence of inactivation was also significantly higher in Na\(_{\alpha, 1.5}\)/delQKP (k\(_{\text{v}}\) = − 5.8 ± 0.2) than in Na\(_{\alpha, 1.5}\)/WT (k\(_{\text{v}}\) = − 7.0 ± 0.2) as shown by the slope of the inactivation curve (Fig. 6D). We next used a double-pulse protocol to directly measure the kinetics of recovery from inactivation. The recovery kinetics, which were determined by fitting a single-exponential equation, were more than two times faster for Na\(_{\alpha, 1.5}\)/delQKP (t\(_{\text{rec}}\) = 3.9 ± 0.3 ms) than for Na\(_{\alpha, 1.5}\)/WT (t\(_{\text{rec}}\) = 9.9 ± 0.9 ms) (Fig. 6E). Na\(_{\alpha, 1.5}\)/WT was slightly slower to recover than the control (t\(_{\text{rec}}\) = 7.1 ± 0.5 ms). The decay of inactivation of the mutated channel was also affected. Na\(_{\alpha, 1.5}/\)delQKP exhibited three times faster inactivation than Na\(_{\alpha, 1.5}\)/WT as calculated by the current decays with an exponential function (Fig. 6F). For example, at − 40 mV, the time constant of decay was 1.1 ± 0.1 ms and 3.0 ± 0.3 ms for Na\(_{\alpha, 1.5}\)/WT and Na\(_{\alpha, 1.5}\)/delQKP, respectively. This faster inactivation rate can be clearly seen on the representative current traces in Fig. 6A. These results indicate that the inactivation process of the channels was compromised by the deletion of the three amino acids. This situation often generates the appearance of a persistent Na\(^+\) current, which is what occurred with the variant channel. Na\(_{\alpha, 1.5}\)/delQKP produced a persistent Na\(^+\) current more than four times larger than Na\(_{\alpha, 1.5}\)/WT, reaching 2.2 ± 0.3%, while it was at 0.5 ± 0.2% of the transient current in Na\(_{\alpha, 1.5}\)/WT (Fig. 6G). The nature of this non-inactivating current was confirmed by its block with 20 µmol/L TTX (Fig. 6H). The biophysical parameters of the voltage-clamp recordings are compiled in Supplementary Table S2 online.

One of the major advantages of our new expression system using Na\(_{\alpha, 1.5}\)/KO iPSC-CMs is the ability to study the impact of the mutation on APs directly. The results described above showed that the Na\(^+\) current was restored but was affected when Na\(_{\alpha, 1.5}\)/delQKP was transfected. We measured the impact of these biophysical effects on APs in current-clamp experiments. The first noticeable effect seen on APs after Na\(_{\alpha, 1.5}\)/delQKP transfection was the increase in APD compared to those recorded in cells transfected with the WT channel. The representative AP traces in Fig. 7A clearly show this increase in duration for Na\(_{\alpha, 1.5}/\)delQKP. The APD\(_{\text{20}}\) and APD\(_{\text{90}}\) of Na\(_{\alpha, 1.5}\)/WT were 81 and 92 ms smaller, respectively, than those of the control. The dV/dt\(_{\text{max}}\), which was compromised by the deletion of the three amino acids. This situation often generates the appearance of a persistent Na\(^+\) current, which is what occurred with the variant channel. Na\(_{\alpha, 1.5}\)/delQKP produced a persistent Na\(^+\) current more than four times larger than Na\(_{\alpha, 1.5}\)/WT, reaching 2.2 ± 0.3%, while it was at 0.5 ± 0.2% of the transient current in Na\(_{\alpha, 1.5}\)/WT (Fig. 6G). The nature of this non-inactivating current was confirmed by its block with 20 µmol/L TTX (Fig. 6H). The biophysical parameters of the voltage-clamp recordings are compiled in Supplementary Table S2 online.

**Discussion**

In this study, we used CRISPR-Cas9 genomic editing tool to successfully produce a novel iPSC cell line in which the SCN5a gene coding for the cardiac Na\(^+\) channel Na\(_{\alpha, 1.5}\) was knocked-out. This iPSC cell line was used to produce cardiomyocytes (iPSC-CMs) that no longer express Na\(_{\alpha, 1.5}\) channels. We first characterized by electrophysiological recordings and optical mapping analysis the effect of the Na\(_{\alpha, 1.5}\) KO on the AP itself and their propagation in iPSC-CMs monolayer. Secondly, we re-introduced by transient transfection a WT Na\(_{\alpha, 1.5}\) channel or a Na\(_{\alpha, 1.5}\) LQT3 variant, delQKP, as a proof-of-concept to study Na\(_{\alpha, 1.5}\)-linked variants in a human cardiomyocyte environment.

Na\(_{\alpha, 1.5}\) KO iPSC-CMs exhibited spontaneous beats with a frequency comparable to the control cell line. Morphologically, we observed that Na\(_{\alpha, 1.5}\) KO iPSC-CMs express MLC2v and troponin T, two essential structural proteins involved in the formation of myosin and actin filaments, respectively. These proteins play an essential role in the organization of sarcomeres, and in the parallel alignment and movement of the filaments. The suppression of Nav1.5 channels obviously did not affect the organized contractile apparatus to modify the contractility capabilities and rhythm of iPSC-CMs.
Figure 6. Altered biophysical properties of Na+ channel in KO iPSC-CMs expressing NaV1.5/ΔQKP. (A) Representative Na+ currents recorded in control and KO iPSC-CMs transfected with NaV1.5/WT or NaV1.5/ΔQKP. The dashed line represents zero current. The currents were obtained using 200 ms pulses from −100 to +40 mV in +5 mV increment. (B) Normalized intensity/voltage relationships (I/V) recorded in control (n = 14), NaV1.5/WT (n = 13) and NaV1.5/ΔQKP (n = 9) iPSC-CMs. Na+ current densities were measured by normalizing current amplitudes to membrane capacitance. Inset shows graph of Imax. (C) Steady-state activation of Na+ currents. Activation curves were generated using a standard Boltzmann distribution: G(V)/Gmax = 1/(1 + exp (−(V − V1/2)/k)). Inset shows graph of V1/2. (D) Steady-state inactivation of control (n = 14), NaV1.5/WT (n = 13) and NaV1.5/ΔQKP (n = 9). Inactivation currents were obtained using 20 ms test pulses to −30 mV after a 500 ms pre-pulse to potentials ranging from −120 to +30 mV. The inactivation values were fitted to a standard Boltzmann equation: I(V)/Imax = 1/(1 + exp ((V − V1/2)/k)) + C. Inset shows graph of V1/2 and k slope. (E) Recovery from inactivation values recorded from control (n = 14), NaV1.5/WT (n = 12) and NaV1.5/ΔQKP (n = 9) iPSC-CMs. The cells were depolarized to −30 mV for 40 ms from a holding potential of −100 mV to inactivate the Na+ channels. Test pulses were then applied at −30 mV for 20 ms to measure current amplitudes, with an interval ranging from 0.1 to 4000 ms. The resulting curves were fitted with a simple exponential equation: (A (exp (− t/τ) + C). Inset shows graph of trec. (F) Persistent Na+ current/voltage relationships recorded in control (n = 9), NaV1.5/WT (n = 11) and NaV1.5/ΔQKP (n = 12) iPSC-CMs. The currents were obtained using 300 ms pulses from −100 to +40 mV in +5 mV increment. Persistent Na+ currents are represented as percentage of the current, obtained by normalizing the values at the end of the pulse to the maximum transient current. Inset shows graph of percentage of current at −35 mV. (H) Representative persistent Na+ current at −35 mV recorded in NaV1.5/WT vs. NaV1.5/ΔQKP using ANOVA and Dunnett's post hoc tests. This figure was made using MICROSOFT POWERPOINT Version 2106 (build 14131.20278 Office, https://docs.microsoft.com/en-us/) and PRISM 8 Version 8.0.2 (263, https://www.graphpad.com/).
As expected, electrophysiological recording revealed a marked 97% reduction in Na\(^+\) currents in the Na\(^+\)V1.5 KO iPSC-CMs. A very small inward and fast-gated Na\(^+\) current remains. By its high sensitivity to TTX, this inward current could probably be carried by Na\(^+\)V1.7 channels which have already been identified by our group as slightly expressed in iPSC-CMs\(^{13}\). Such a reduction in Na\(^+\) currents should have a major impact on the APs given their role in the triggering and the rising phase (phase 0) of the APs. Furthermore, the entry of Na\(^+\) in the cardiomyocytes is normally followed by a cascade of ion channels activation such as K\(^+\) and Ca\(^{2+}\) channels that contribute to the plateau and the repolarisation phase of APs\(^{14}\). We can immediately notice that APs were still elicited in Na\(^+\)V1.5 KO iPSC-CMs. Their resistance to TTX (1 µmol/L) but their complete inhibition by nifedipine (1 µmol/L) reveal the Ca\(^{2+}\) nature of the APs in the KO cells. These Ca\(^{2+}\) APs were characterized by a rising phase (phase 0) considerably slow due to the lack of Na\(^+\) channel, as calculated by the dV/dt\(_{\text{max}}\) from the electrophysiological recordings. Moreover, a stronger injected current was required to trigger Ca\(^{2+}\) APs. Normally, during the phase 0, Na\(^+\) channels contribute to more depolarization and the opening of Ca\(^{2+}\) channels. In our situation where there are few Na\(^+\) channels, a stronger depolarization is required to open Ca\(^{2+}\) channels. These alterations will certainly impact the propagation of the electrical signal through the myocardium.

We take advantage of the properties of iPSC-CMs to form electrical coupled syncytia of monolayers and we have indeed found a drastically slowing of the conduction velocity in the KO. The role of Na\(^+\)V1.5 in the cardiac conduction was already known as shown in a previous study with heterozygous (Scn5a\(^{+/−}\)) Na\(^+\)V1.5 mice. These mice exhibited a 50% reduction in Na\(^+\) current and had impaired atrioventricular conduction and delayed intra myocardial conduction\(^{15}\). Our optical mapping experiments confirmed the critical role of Na\(^+\)V1.5 for controlling the speed of electrical wave propagation trough myocytes. The slowing of conduction velocity could be related to
the lack of Na+ channels in the intercalated discs, but, probably to connexins dysregulation, where their role must be studied in the context of Na+,1.5 KO. It is known that connexins interact physically with Na+ channels and the lack of these channels in the intercalated discs may impede their role in establishing a normal conduction velocity.

Another major parameter involved was the duration of the action potential. We found contradictory results according to the experiments carried out. In electrophysiological recordings, the duration of the AP was found to be prolonged while this was shortened in optical mapping analysis in the Na+,1.5 KO iPSC-CMs. In cardiomyocytes, Na+,1.5 influence the duration of the AP by the non-inactivating channels that re-open and generate a small inward current (persistent Na+ current) during the plateau phase. Previous studies have shown that TTX, a Na+ channel blocker, shortened the APD in dog and guinea pig ventricle heart muscle but not in atrial. In addition, heterozygous (Scn5aΔ/+), Na+,1.5 mice showed shorter APDs in the right ventricular epicardium but not in the left ventricular studies with other Na+ blockers such as flecainide, quinidine and lidocaine showed that the APD can also be increased depending on the stimulation frequency, tissue location and species. To resume, the effect of reduction or the suppression of Na+,1.5 expression on APDs is not straightforward but influenced by the tissue and the Na+ channel blocker used, among other factors. In this study, the results disparity between patch-clamp and optical mapping experiments could originate from the nature of the sample. In one case, the recordings were done in individual cells whereas in the other case they were performed on monolayers as behave as a functional syncytium. This highlights the complexity of the contributing factors that can influence the duration of the action potential. Further studies are warranted to understand more this difference.

To complete the Na+,1.5 KO iPSC-CMs characterization, we were interested to Ca2+ homeostasis by measuring the Ca2+ currents by the Patch-clamp technique and the propagation of Ca2+ transients by optical mapping. The absence of Na+,1.5 does not modify the biophysical properties of L-type Ca2+ channels but altered the propagation of Ca2+ transients. Ca2+ transients represent Ca2+ release/reuptake cycles that coordinate contraction and relaxation of cardiomyocytes. Even though Na+,1.5 KO iPSC-CMs beat spontaneously and at rhythm similar to control, Ca2+ propagation velocity has significantly decreased. This result is perfectly correlated with the reduction in conduction velocity and could be explained by alteration of the excitation–contraction coupling in the absence of Na+,1.5 in cardiomyocytes. The intracellular Ca2+ release is triggered by the AP, which conduction is delayed in the KO, which therefore also causes the observed delay in the wave of Ca2+ release. Optical recordings on Na+,1.5 KO iPSC-CMs also showed a decrease in amplitude of Ca2+ transients signal, probably indicating a reduction of Ca2+ availability at each cycle. This could be explained by other Ca2+ handling players such as Ryanodine channel or SERCA pump.

In the second part of this study, we have re-introduced by DNA transfection the Na+,1.5 WT channel or the Na+,1.5 delQKP LQT3 variant in the Na+,1.5 KO iPSC-CMs and compared their biophysical effects. The transfection of the Na+,1.5 WT channel in KO iPSC-CMs successfully restored Na+ channel expression and the fast upstroke velocity of APs. In fact, the upstroke velocity was even faster in Na+,1.5 WT than in control. We hypothesize that this difference could be explained by the higher level of Na+,1.5 channels due to its overexpression according to the experiments carried out. In electrophysiological recordings, the duration of the AP was found to be prolonged at the cellular level results in a prolongation of the QT interval on the electrocardiogram of patients with LQT syndrome. Moreover, patients are at increased risk for the development of polymorphic ventricular tachycardia, specifically torsade de pointes (TdP). Thanks to our model, we were able to show that the delQKP variant induces a prolongation of APD at the origin of LQT, and more interesting, causes EADs, responsible for TdP.

Interestingly, during electrophysiological recordings, we observed differences between the control and Na+,1.5 KO iPSC-CMs transfected with Na+,1.5/delQKP exhibit the essential LQT3 features. Their APs presented a delayed and a prolonged repolarization phase, leading to arrhythmic events such as EADs and DADs. The prolongation of the AP is most likely caused by the increase in the persistent Na+ current features. Their APs presented a delayed and a prolonged repolarization phase, leading to arrhythmic events such as Ryanodine channel or SERCA pump.
differentiation to cardiomyocytes has increased considerably with novel differentiation protocols, the level of iPSC-CM maturity remains an issue. This low level of maturity is characterized by the expression of numerous fetal isoforms to the detriment of adult isoforms, including those of Na\textsubscript{v}1.5\textsuperscript{28}. Thereby, when we compared the transfected Na\textsubscript{v}1.5/WT with the control iPSC-CMs, in fact we mostly compared the effects between the adult and the fetal isoforms. However, when we compared the transfected Na\textsubscript{v}1.5/WT with the transfected Na\textsubscript{v}1.5/delQKP both channels were in the adult isoform background. This is an additional advantage of the new Na\textsubscript{v}1.5 KO iPSC-CM, i.e., the ability to study Na\textsubscript{v}1.5 variants in the desired background, either adult or fetal isoforms.

**Conclusion**

In conclusion, thanks to our Na\textsubscript{v}1.5 KO iPSC model, we were able first to characterize the impact of the KO of Na\textsubscript{v}1.5 on APs as well as on the propagation of voltage and Ca\textsuperscript{2+} waves on iPSC-CMs. Secondly, as a proof-of-concept, we were able to directly study the impacts on the APs of a known mutation linked to LQT3. The workflow of our novel approach is straightforward. The iPSC-CM from the Na\textsubscript{v}1.5 KO cell line can be frozen and thawed on demand to study any Na\textsubscript{v}1.5 variants by a simple transfection, as easily than with an expression system such as HEK293 cells with however several advantages as described above. Na\textsubscript{v}1.5 variants and drug effects can now be straightforwardly and easily studied in a cardiac cellular environment. We hope that in the future, our new tool will help in the diagnosis and treatment of patients suffering of cardiovascular diseases.

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Author contributions
M.P. acquisition of data, analysis, interpretation of data, drafting of the manuscript. M.D. acquisition of data, analysis, interpretation of data, drafting of the manuscript. H.P. acquisition of data, analysis, interpretation of data, drafting of the manuscript. M.C. study concept and design, study supervision, interpretation of data, drafting and critical revision of the manuscript. All authors reviewed the manuscript before its submission. All the figures were made by the co-authors.

Competing interests
The authors declare no competing interests.

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