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Lab Resource: Genetically-Modified Single Cell Line

Generation of a heterozygous *SCN5A* knockout human induced pluripotent stem cell line by CRISPR/Cas9 edition

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A B S T R A C T

Mutations leading to haploinsufficiency in *SCN5A*, the gene encoding the cardiac sodium channel $Na_v1.5$ α-subunit, are involved in life-threatening cardiac disorders. Using CRISPR/Cas9-mediated genome edition, we generated here a human induced-pluripotent stem cell (hiPSC) line carrying a heterozygous mutation in exon 2 of *SCN5A*, which leads to apparition of a premature stop codon. SCN5A-clone 5 line maintained normal karyotype, morphology and pluripotency and differentiated into three germ layers. Cardiomyocytes derived from these hiPSCs would be a useful model for investigating channelopathies related to *SCN5A* heterozygous deficiency.

1. Resource table

Unique stem cell line identifier	1. ICANi002-A-2 *https://hpscreg.eu/cell-line/ICANi002-A-2*
Alternative name(s) of stem cell line	SCN5A-clone 5
Institution	Sorbonne Université, Inserm, Research Unit on Cardiovascular and Metabolic Diseases, UMRS-1166, F-75013 Paris, France
Contact information of the reported cell line distributor	Nathalie Neyroud: nathalie.neyroud@sorbonne-universite.fr
Type of cell line	iPSCs
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 30 Sex: Male Ethnicity: Caucasian
Cell Source	Skin fibroblasts
Method of reprogramming	Non-integrative episomal plasmid reprogramming
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	PCR negative for episomal reprogramming plasmid backbones

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Unique stem cell line identifier	1. ICANi002-A-2 *https://hpscreg.eu/cell-line/ICANi002-A-2*
Cell culture system used	Feeder-free conditions
Type of Genetic Modification	Induced frameshift mutation in *SCN5A* leading to the apparition of a premature stop codon
Associated disease	Brugada syndrome OMIM # 601144 Progressive Familial Heart Block, type IA OMIM # 113900
Gene/locus	*SCN5A*, 3p22.2
Method of modification/site-specific nuclease used	CRISPR/Cas9 induced non-homologous end-joining (NHEJ)
Site-specific nuclease (SSN) delivery method	Plasmid pX459v2.0-SpCas9-HF1 nucleofection
All genetic material introduced into the cells	Reprogramming plasmids pCXLE-hUL, pCXLE-hOCT3/4 and pCXLE-hSK (Addgene) Genome edition plasmid pX459V2.0-SpCas9-HF1 (Addgene) carrying Cas9 and the sgRNA targeting exon 2 of *SCN5A*
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield photography	Typical morphology of human pluripotent stem cell	Fig. 1C
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Positive for SOX2, OCT4, NANOG, SSEA-4, TRA-1-60 and TRA-1-81	Fig. 1D
	Quantitative analysis (RT-qPCR)	Positive for SOX2, OCT3/4, NANOG and CRYPTO	Fig. 1E
Karyotype	Karyotype using m-FISH	46XY	Fig. 1B
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	Heterozygous status	Sanger sequencing profiles are shown Fig. 1A
	Transgene-specific PCR	Absence of transgene integration	Supplementary_S2
Verification of the absence of random plasmid integration events	PCR amplification of plasmid backbones	Absence of plasmid integration	Supplementary_S2
Parental and modified cell line genetic identity evidence	STR analysis	Homo sapiens (human), 18 loci tested, all matched between lines and donor fibroblasts	STR analysis
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	1 bp deletion, heterozygous	Sanger sequencing is shown on Fig. 1A
	PCR-based analyses	N/A	N/A
	Western blotting	Western-blot revealed a reduced Nav1.5 protein expression in SCN5A-Clone 5	Fig. 1I
Off-target nuclease analysis	PCR across the top 10 off-target sites	Absence of unexpected variants in the top-10 predicted off-target sites	Supplementary_S1
Specific pathogen-free status	Mycoplasma	Negative	Supplementary_S3
Multilineage differentiation potential	Embryoid body and Scorecard	Differentiation into all three germ layers confirmed by scorecard performed on RNA from embryoid bodies	Fig. 1F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

(*continued*)

Unique stem cell line identifier	1. ICANi002-A-2 *https://hpscreg.eu/cell-line/ICANi002-A-2*
Method of the off-target nuclease activity surveillance	PCR /sequencing of 10 off-target sites
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Episomal puromycin resistance in genome edition plasmid pX459V2.0-SpCas9-HF1
Inducible/constitutive system details	N/A
Date archived/stock date	September 2021
Cell line repository/bank	UMRS-1166-iPS-ICAN repository
Ethical/GMO work approvals	Original human fibroblasts purchased from PromoCell (Germany) See www.promocell.com/ethics
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	Genome edition plasmid pX459V2.0-SpCas9HF1 was a gift from Yuichiro Miyaoka (Addgene plasmid #108293; http://n2t.net/addgene: 108293; RRID: Addgene_108293) Reprogramming plasmid pCXLE-hUL was a gift frome Shinya Yamanaka (Addgene plasmid # 27080; http://n2t.net/addgene:27080; RRID:Addgene_27080) Reprogramming plasmid pCXLE-hOCT3/4-shp53-F was a gift from Shinya Yamanaka (Addgene plasmid # 27077; http://n2t.net/addgene:27077; RRID: Addgene_27077) Reprogramming plasmid pCXLE-hSK was a gift from Shinya Yamanaka (Addgene plasmid # 27078; http://n2t.net/addgene:27078; RRID:Addgene_27078)

2. Resource utility

Expression and/or function of the cardiac sodium channel $Na_v1.5$, encoded by *SCN5A*, are impaired in hereditary arrhythmias. The SCN5A-clone 5 cell line was established to provide a relevant model of sodium channelopathies related to *SCN5A* haploinsufficiency and an excellent human cellular tool for drug screening and for developing novel therapy (Table 1).

3. Resource details

The cardiac voltage-gated sodium channel α-subunit $Na_v1.5$, encoded by *SCN5A*, plays a key role in cardiac excitability, as emphasized by more than 250 pathogenic variants identified in *SCN5A* and related to cardiac arrythmias transmitted as autosomal-dominant traits (Wilde and Amin, 2018). Since animal models have inherent limits due to the fundamental differences in cardiac electrophysiology between mice and humans and because human adult cardiomyocytes are not obtainable, *in vitro* human models are needed to explore cardiac-arrythmia pathophysiology. With the aim to model a deficit in $Na_v1.5$ in human cells as it is observed in patients affected by some loss-of-function mutations in *SCN5A*, we engineered the hiPSC line SCN5A-clone 5 carrying a heterozygous *SCN5A* mutation, leading to apparition of a premature stop codon and haploinsufficiency, by CRISPR-Cas9 genome edition (Ran et al., 2013) of the control hiPSC line ICAN-403.3 (ICANi002-A in hPSCreg: https://hpscreg.eu/cell-line/ICANi002-A).

The pX459V2.0-SpCas9-HF1 plasmid carrying the Cas endonuclease sequence and a specific guide RNA (sgRNA) targeting the start codon in exon 2 of *SCN5A* was electroporated in the control hiPSC line to induce non-homologous end-joining (Fig. 1A). After Sanger sequencing, the puromycin-resistant clone SCN5A-clone 5 was selected, harboring the *SCN5A* heterozygous frameshift mutation Chr3(GRCh38): g.38633263del (NM-198056.2 (SCN5A):c.45del; p.Arg15Serfs*82), generating a premature stop codon at amino-acid 96 of $Na_v1.5$ (Fig. 1A). This genome edition targeting the start codon of *SCN5A* reproduces the $Scn5a^{+/-}$ murine model (Papadatos et al., 2002). $Na_v1.5$ expression was analyzed by western-blot and the reduction of the protein expression observed in SCN5A-clone 5 cardiomyocytes confirmed *SCN5A* deficiency in this hiPSC line (Fig. 1I). The absence of mutations in the predicted top-10 off-target sites was assessed by PCR/sequencing (Supplementary_S1) and the non-integration of episomal plasmids was validated by PCR targeted on plasmid backbones (Supplementary_S2) (Okita et al., 2011). Short tandem repeat (STR) analysis of 18 loci demonstrated matching genetic identities between SCN5A-clone 5, control hiPSC ICAN-403.3 and donor fibroblasts (STR analysis). M-fish cytogenetic analysis performed on SCN5A-clone 5 showed a normal karyotype (Fig. 1B).

$SCN5A^{+/-}$ cells exhibited a typical hiPSC morphology, similar to control hiPSCs (Fig. 1C) and were tested negative for mycoplasma

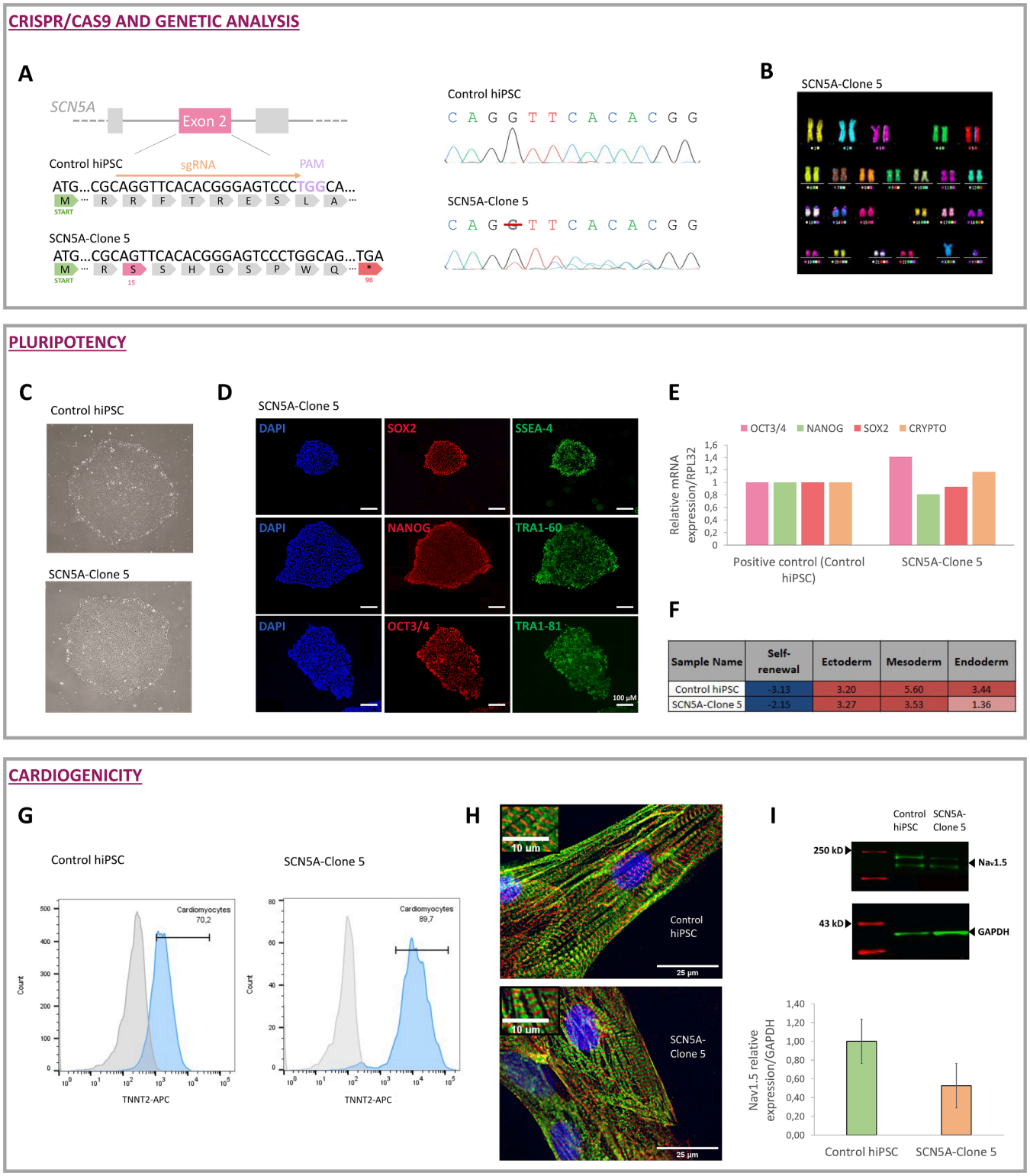

Fig. 1. Characterization and differentiation of ICANi002-A-2 line. (A) CRISPR/Cas9 genome edition. (B) Karyotype. (C) Brightfield images of control and edited iPSC lines. (D) Immunostaining and (E) RT-qPCR of pluripotency markers. (F) Scorcard analysis. (G) Percent of Troponin-T positive cells counted by cytometry analysis. (H) Immunostaining of α-actinin (red) and troponin T (green) in cardiomyocytes derived from iPSC lines. (I) $Na_v1.5$ relative-expression quantification by Western-blot.

(Supplementary_S3). Furthermore, SCN5A-clone 5 hiPSCs expressed nuclear (OCT3/4, SOX2, NANOG) and transmembrane (TRA1-60, TRA1-81, SSEA-4) pluripotency markers, as demonstrated by immunofluorescence staining (Fig. 1D) and by RT-qPCR (OCT3/4, SOX2, NANOG and CRYPTO), which showed expression levels comparable to control hiPSCs (Fig. 1E). In parallel, a scorecard analysis was performed on RNA extracted from embryoid bodies to assess the trilineage differentiation potential of SCN5A-clone 5, showing that the *SCN5A*$^{+/-}$ hiPSCs retained the ability to differentiate spontaneously into all three germ layers with an extinction of pluripotency marker expression (Fig. 1F).

Table 2
Reagents details.

Antibodies used for immunocytochemistry, western-blot and flow cytometry:			
Assays	Antibody	Company Cat# and RRID	Condition
Pluripotency markers (Immunostaining)	Rabbit anti-OCT4	Biovision-Cliniscience Cat # 3576-100 RRID: Ab_2167563	1/200 in 1/10 blocking solution
	Rabbit anti-SOX2	Millipore Cat # AB5603 RRID: AB_2286686	1/200 in 1/10 blocking solution
	Rabbit anti-NANOG	Cell signaling Ozyme Cat # 4903S RRID: AB_10559205	1/200 in 1/10 blocking solution
	Mouse anti-SSEA4	Santa Cruz Cat #sc21704 RRID: AB_628289	1/100 in 1/10 blocking solution
	Mouse anti-TRA-1-60	Millipore Cat # MAB4360 RRID: AB_2119183	1/100 in 1/10 blocking solution
	Mouse anti-TRA-1-81	Millipore Cat # MAB4381 RRID: AB_177638	1/100 in 1/10 blocking solution
Differentiation markers (Immunostaining)	Rabbit anti-troponin T	Abcam Cat # ab45932 RRID: AB_956386	1/500 in 1/10 blocking solution
	Mouse anti-α-actinin	Sigma Aldrich Cat # A7811 RRID: AB_476766	1/1000 in 1/10 blocking solution
Nuclear staining	DAPI	Sigma Aldrich Cat # D9542	1/10000 in 1/10 blocking solution
Secondary antibodies (Immunostaining)	Alexa fluor 594 chicken anti-rabbit IgG	Thermo Fisher Scientific Cat # A21442 RRID: AB_2535860	1/1000 in 1/10 blocking solution
	Dylight 488 goat anti-mouse IgG	Bethyl Cat # A90-116D2 RRID: AB_10631770	1/1000 in 1/10 blocking solution
Mounting media	Dako fluoromount	Dako #S3023	–
Flow cytometry	Anti-cardiac Troponin T antibody, REAfinity™ APC	Miltenyi Cat # 130-120-543 RRID: AB_2783888	1/200 in Inside perm solution
	REA control antibody, APC, REAfinity™	Miltenyi Cat # 130-113-446 RRID: AB_2733446	1/200 in Inside perm solution
Primary antibodies (Western-blot)	Rabbit anti-$Na_v1.5$	Alomone Labs Cat #ASC-005 RRID: AB_2040001	1/200 in PBS-Tween 0.1%-Milk 5%
	Mouse anti-GAPDH	Proteintech Cat #60004-1-Ig RRID: AB_2107436	1/10000 in PBS-Tween 0.1%-Milk 5%
Secondary antibodies (Western-blot)	IR dye 800 donkey anti-rabbit	Li-Cor Biosciences #92632213 RRID: AB_621848	1/10000 in PBS-Tween 0.1%-Milk 5%
Primers and Oligonucleotides:			
Assays	Target	Forward and reverse primer (5′-3′)	Condition
sgRNA	SCN5A	5′ GGGACTCCCGTGTGAACCTG 3′	–
Targeted mutation sequencing	SCN5A	5′ GAATCAGGCCCATTGTCTGT 3′ 5′ GTGACTCATTTCCCCAGAGC 3′	Tm 60 °C 40 cycles 491 pb
Edition plasmid integration (PCR)	AmpR	5′ GCTATGTGGCGCGGTATTAT 3′ 5′ AAGTTGGCCGCAGTGTTATC 3′	Tm 60 °C 35 cycles 186 pb
Reprogramming plasmid integration (PCR)	EBNA1	5′ ATCAGGGCCAAGACATAGAGATG 3′ 5′ GCCAATGCAACTTGGACGTT 3′	Tm 60 °C 35 cycles 61 pb
Pluripotency markers (qPCR)	SOX2	5′ CCCAGCAGACTTCACATGT 3′ 5′ CCTCCCATTTCCCTCGTTTT 3′	Tm 60 °C 40 cycles 151 pb
	NANOG	5′ CTCCAACATCCTGAACCTCAGC 3′ 5′ CGTCACACCATTGCTATTCTTCG 3′	Tm 60 °C 40 cycles 115 pb
	OCT4	5′ CCTCACTTCACTGCACTGTA 3′ 5′ CAGGTTTTCTTTCCCTAGCT 3′	Tm 60 °C 40 cycles 164 pb
	CRYPTO	5′ACAGAACCTGCTGCCTGAAT 3′ 5′ATCACAGCCGGGTAGAAATG 3′	Tm 60 °C 40 cycles 215 pb
House-keeping gene (qPCR)	RPL32	5′ AGTTCCTGGTCCACAACGTC 3′ 5′ GTGACTCTGATGGCCAGTTG 3′	Tm 60 °C 40 cycles 142 pb
Off-target analysis (PCR)	OFF-TARGET 1	5′ AAAGGTGGGTGGGAATATTTG 3′ 5′ TCCCGTTGACAGGGATTAAA 3′	Tm 60 °C 35 cycles 226 pb
	OFF-TARGET 2	5′ TTCAAGCAAGTGCCAAGTATG 3′ 5′ TGCTCATTCTCCATGGCTTT 3′	Tm 60 °C 35 cycles 225 pb
	OFF-TARGET 3	5′ GAGCATTTCCTACACTCGGC 3′ 5′ CCTGTACATGCCTTCCCTCA 3′	Tm 60 °C 35 cycles 246 pb
	OFF-TARGET 4	5′ CTGTTTGTTCCCTAAGGCGG 3′ 5′ GCGTGCAACCTAGATCCTTG 3′	Tm 60 °C 35 cycles 198 pb
	OFF-TARGET 5	5′ TTCTGATTGAACACTGCCGC 3′ 5′ AATGGTGTGGGAGAGGTTGT 3′	Tm 60 °C 35 cycles 192 pb
	OFF-TARGET 6	5′ CCACTGCCAATATCACCAGA 3′ 5′ GGGCCGAGACGAATACATAG 3′	Tm 60 °C 35 cycles 221 pb
	OFF-TARGET 7	5′ ACCCAGATCATAGGCAGGTG 3′ 5′ CCTGTATGCTGGGTCCTGTT 3′	

(*continued on next page*)

Table 2 (*continued*)

Antibodies used for immunocytochemistry, western-blot and flow cytometry:			
Assays	Antibody	Company Cat# and RRID	Condition
			Tm 60 °C 35 cycles 207 pb
	OFF-TARGET 8	5′ TGGTGGTTACTCTCTCGGTTG 3′ 5′ CCTCTGCAGGACATCTGGTT 3′	Tm 60 °C 35 cycles 206 pb
	OFF-TARGET 9	5′ CCATCCCCTTCCTGGTAGTC 3′ 5′ CTGAGAGGTGACACAGTGGA 3′	Tm 60 °C 35 cycles 151 pb
	OFF-TARGET 10	5′ TTCACCCCAGGCCAAAGTAT 3′ 5′ TCTCTTGGAGCATATGAAGGC 3′	Tm 60 °C 35 cycles 218 pb

Cardiogenicity of SCN5A-clone 5 was evaluated after differentiation of hiPSCs in 2D culture according to the protocol based on small molecules modulation of the Wnt/β-catenin pathway (Lian et al., 2013). Importantly, the edited line efficiently differentiated into beating cardiomyocytes with 90% purity according to FACS quantification (Fig. 1G), showing normal sarcomere morphology, as assessed by immunostaining of α-actinin (red signal) and troponin T (green signal) (Fig. 1H).

4. Materials and methods

4.1. Cell culture and differentiation

hiPSCs were cultured on matrigel-coated plates in mTeSR™1 (*STEMCELL Technologies)* at 37 °C and 5% CO_2 and manually passaged every 4–6 days. Detection of mycoplasma was performed by *Eurofins genomic* (Mycoplasmacheck).

Human iPSCs were dissociated using Gentle cell reagent (*STEMCELL Technologies*) and plated in mTeSR™1 supplemented with 10-μM Y-27632 (Biotechne) for 24 h. Cells were then differentiated into cardiomyocytes as previously described (Lian et al., 2013).

4.2. Genome edition

The selected sgRNA targeting *SCN5A* (Table 2) predesigned on https://genome.ucsc.edu/ was cloned into pX459V2.0-SpCas9HF1 (*Addgene*) and electroporated into control hiPSCs using the Amaxa Nucleofector™ starter kit (*Lonza*). After 48 h of puromycin selection, single clones were picked, expanded and screened by sequencing (*Eurofins genomic*) of PCR products spanning the target site (Table 2).

4.3. Genomic integrity control

The absence of random episomal plasmid integration was achieved by PCR on gDNA using primers designed on vector backbones (Table 2). The 10 first predicted off-target sites from https://genome.ucsc.edu/ were checked by PCR/sequencing (primer sequences in Table 2). SCN5A-clone 5 karyotype was assessed using the m-fish technique by CECS/I-STEM (*Corbeil-Essonnes, France*) on 40 metaphases. Cell identity was checked on gDNA from donor fibroblasts, control iPSC line ICAN-403.3 and SCN5A-clone 5 by short tandem repeat (STR) analysis of 18 loci (*LGC Standards ATCC*).

4.4. Pluripotency characterization

Total RNA was extracted using RNeasy® mini kit (*Qiagen*) and cDNA was synthetized by Superscript II First Strand Synthesis System (*Invitrogen*). Pluripotency gene expression was assessed by RT-qPCR (Table 2) and normalized to RPL32.

For immunostaining, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized in blocking solution (PBS 2% BSA, 1% Triton) for 30 min and stained with primary antibodies (Table 2) overnight at 4 °C. Then, cells were washed 3 times with PBS and incubated 1 h with secondary antibodies and DAPI for nuclear staining (Table 2), at room temperature. Images were acquired with an epifluorescence Nikon Eclipse Ti microscope (*NIS Elements software*) on Fig. 1D and with a DeltaVision microscope (*GE Healthcare*) on Fig. 1H.

4.5. Trilineage differentiation

To form embryoid bodies, hiPSCs were passaged using a cell scraper into Nuclon™ Sphera™ dishes and cultured in TeSR™ E6 (*STEMCELL Technologies*). Ten days later, RNA was isolated and reverse transcribed. A Taqman™ hPSC Scorecard™ kit (*Thermo Fisher Scientific*) was used to assess the trilineage differentiation potential, according to manufacturer's instructions.

4.6. Flow cytometry

The single cell suspension obtained using Multi Dissociation kit 3 (*Miltenyi Biotec*) was incubated with a viability marker (*Molecular Probes*) for 30 min at 4 °C. Permeabilization, fixation and labelling with anti-TNNT2 antibody (Table 2) were then conducted following the Inside stain kit protocol (*Miltenyi Biotec*). Samples were processed for FACS analysis on a MACSquant analyser 10, (*Miltenyi Biotec*) and analyzed using the FlowJo software.

4.7. Western-blot

Total proteins were extracted from cardiomyocytes using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton). After SDS-PAGE electrophoresis on 7%-Tris-acétate gel (*Thermofisher*), proteins were transferred to a nitrocellulose membrane (*Biorad*) before incubation with antibodies (Table 2). Signal was detected using a Li-COR Odyssey Infrared Imaging system (*Licor*).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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