Modeling of dilated cardiomyopathy by establishment of isogenic human iPSC lines carrying phospholamban C25T (R9C) mutation (UPITII002-A-1) using CRISPR/Cas9 editing

Robert J. Barndt¹, Ning Ma², Ying Tang², Michael P. Haugh³, Laila S. Alamri³, Stephen Y. Chan²,³, Haodi Wu²,³,⁴,*

¹Pittsburgh Heart, Lung, Blood Vascular Medicine Institute, University of Pittsburgh School of Medicine, PA, USA
²Bioland Laboratory (Guangzhou Regenerative Medicine and Health Guangdong Laboratory), Guangzhou, Guangdong 510320, China
³Department of Bioengineering, University of Pittsburgh Swanson School of Engineering, PA, USA
⁴Division of Cardiology, Department of Medicine, University of Pittsburgh School of Medicine, PA, USA

Abstract

As the most common cause of heart failure, dilated cardiomyopathy (DCM) is characterized by dilated ventricles and weakened contractile force. Mutations in the calcium handling protein phospholamban (PLN) are known to cause inherited DCM. Here, we introduced a PLN-R9C mutation in a healthy control induced pluripotent stem cell (iPSC) line using CRISPR/Cas9. The genome-edited iPSC line showed typical pluripotent cell morphology, robust expression of pluripotency markers, normal karyotype, and the capacity to differentiate into all three germ layers in vitro. The PLN-R9C iPSC line provides a valuable resource to dissect the molecular mechanisms underlying PLN mutation-related DCM.

1. Resource utility

Phenotypic variability of different PLN mutations impedes our understanding of their pathomechanisms. Here, the heterozygous PLN-R9C iPSC and its isogenic control line provide unlimited sources of differentiating iPSC-derived cardiomyocytes (iPSC-CMs),

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*Corresponding author at: 200 Lothrop Street, BST E1256, Pittsburgh, PA 15261, USA. haodi@pitt.edu (H. Wu).
These authors contributed equally.

Declaration of Competing Interest
SYC has served as a consultant for United Therapeutics; SYC has held research grants from Actelion and Pfizer. SYC is a director, officer, and shareholder of Synhale Therapeutics. The other 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.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102544.
which are powerful research tools for disease modeling, mechanistic study, and drug development of PLN-R9C mutation-related DCM.

2. Resource details

About 35–40% of DCM cases are hereditary. To date, rare variants in more than 100 genes, including the calcium handling protein phospholamban (PLN), have been identified as DCM mutations (McNally and Mestroni, 2017). PLN is a small protein that inhibits the calcium pump (sarcoplasmic reticulum Ca\(^{2+}\) ATPase, SERCA2a) activity, which is key to the Ca\(^{2+}\) recycling of beating cardiomyocytes. β-adrenergic stimulation and protein kinase A (PKA) mediated phosphorylation can relieve the inhibition on SERCA2a by PLN and significantly enhance cardiac contractile function. Thus, PLN is a key regulator of Ca\(^{2+}\) recycling and β-adrenergic response in the heart. Many PLN genetic variants have been identified as mutations in cardiac diseases, which are highly variable in relevant disease phenotype and severity (Karim et al., 2006; Liu et al., 2015). Among them, R9C is known to cause early-onset severe DCM (Schmitt et al., 2003). Although patient-specific iPSCs have been successfully used as research platforms of DCM (Wu et al., 2015), the lack of isogenic iPSC DCM models has limited our understanding of the pathological mechanisms. To address the need, our study aims to generate PLN-R9C iPSCs as a new disease model using CRISPR/Cas9 single nucleotide editing technology (Tables 1 and 2).

To introduce the PLN-R9C (C25T) mutation into the control line with CRISPR/Cas9, we designed a homologous sgRNA and single-stranded oligo donor (ssODN) that target the DNA regions close to C25 in PLN exon 2 (Fig. 1A). The sgRNA was constructed in pX459 (Addgene #62988) and was co-transfected with the ssODN into control iPSCs. After two days of recovery, the iPSCs were selected with puromycin, and single-cell-derived surviving colonies were collected and expanded. The genome-editing results were validated by genotype sequencing (Fig. 1B). Phase contrast microscopic imaging showed PLN-R9C iPSCs have typical stem cell-like morphology (Fig. 1C), while immunofluorescence staining, flow cytometry (FACS), and real-time PCR analysis suggested strong expression of pluripotent marker genes in the genome-edited iPSCs (Fig. 1D–F). Karyostat examination of the PLN-R9C iPSC line has confirmed regular chromosomal copy number (Fig. 1G). The identical genetic background of both PLN-R9C and the isogenic control (Ctrl) iPSC was confirmed with Cell ID analysis (Fig. 1H). In response to directed three-germ layer differentiation, the genome-edited iPSCs showed robust expression of germ layer-specific markers by immunofluorescence staining, such as TUBB3 for ectoderm, BRACHYURY for mesoderm, and SOX17 for endoderm (Fig. 1I). Mycoplasma PCR showed a negative result in both Ctrl and PLN-R9C iPSCs. (Supplementary Fig. 1A) Also, PCR confirmed there is no genomic integration of pX459 backbone in PLN-R9C iPSCs. (Supplementary Fig. 1B) To exclude the possibility of genetic mutations induced by off-target Cas9 activity, the sequence of the top five predicted sgRNA off-target sites (Supplementary Fig. 1C) was unchanged in the genome-edited iPSCs (Supplementary Fig. 1D).

In summary, the genome-edited PLN-R9C iPSCs showed typical morphology, pluripotency marker expression, and differentiation capacity of embryonic stem cells. The PLN-R9C
iPSC line and its isogenic control provide valuable resources to understand the pathological mechanisms of DCM and develop novel therapeutic tools.

3. Materials and methods

3.1. Maintenance of iPSC culture

The human iPSC lines were maintained with daily Essential 8 (E8) media change (Thermo Fisher Scientific) on Matrigel-coated plates (Corning) at 37 °C with 5% CO₂ and 5% O₂. For passaging, iPSCs were dissociated using 0.5 μM EDTA in DPBS (Life Technologies) and were split 1:10 in E8 with 10 μM Y-27632 dihydrochloride (Med Chem Express).

3.2. Gene editing with CRISPR/Cas9

The PLN-sgRNA oligos and the ssODN were designed using online CRSIPR designing tools (https://benchling.com/). The sgRNA was cloned into pSpCas9(BB)-2A-Puro (pX459) V2.0 (Addgene plasmid #62988). Purified pX459-PLN-sgRNA (2 μg) and ssODN (4 μg) were transfected into 1 × 10⁶ Ctrl iPSCs at 30–40% confluency using Lipofectamine 3000 (Thermo Scientific). After recovery for 2 days, the iPSCs were treated with 1 μg/ml Puromycin (Life Technologies) for 24 h. After 2–3 weeks, surviving single iPSC colonies were manually picked and transferred to 24-well plates. The colonies with successful gene editing were identified by genotyping PCR after expansion.

3.3. DNA extraction and genotyping

Genomic DNA was isolated using QuickExtract DNA Extraction Solution (Lucigen). The PLN gene was amplified using Platinum PCR SuperMix High Fidelity (Invitrogen), and the PCR products were column purified (Zymo Research) prior to genotyping by Sanger sequencing. For the off-target analysis, the genomic DNA of PLN-R9C iPSCs was amplified with off-target primer sets (Table 2) and Q5 High-Fidelity 2X Master Mix (NEB), and the PCR products were purified with QIAquick gel extraction kit (Qiagen) and ligated into the pMiniT 2.0 vector (NEB) for sequencing. The sequencing data was visualized using SnapGene 5.3.

3.4. RNA extraction and real-time PCR

RNA was isolated using TRIzol (Ambion-Life Technologies), treated with DNase I for 30 min, and then purified using Direct-zol RNA MicroPrep columns (Zymo Research). The cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad), and the pluripotency marker genes (Table 2) were quantified with iTaq Universal SYBR Green Supermix (Bio-Rad) on a StepOnePlus analyzer (Applied Biosystems-Life Technologies).

3.5. Flow cytometry

The PLN-R9C iPSCs were stained with TRA-1–60(S) antibody (Table 2). Labeled cells were analyzed with the LSR Fortessa flow cytometer (BD) with 488 100mW Octagon laser, 505 nm LP mirror, and 530/30 BP filter. The FACS data was quantified using FlowJo software.
3.6. Differentiation of three germ layers

The PLN-R9C iPSCs were differentiated into three germ layers (ectoderm, mesoderm, endoderm) using STEMdiff™ Trilineage media (StemCell Technologies) and labeled with germ-layer specific antibodies (Table 2).

3.7. Immunofluorescence labeling

Cells were fixed with 4% PFA (Sigma) for 15 min at room temperature, permeabilized with 0.2% Triton X-100/1% BSA (Sigma) for 1 h and blocked with 5% BSA for 2 h. Primary antibodies (Table 2) were incubated with 0.1% TritonX-100/1% BSA overnight at 4 °C, followed by the incubation with secondary antibodies for 1.5 h at room temperature. Nuclei were stained with 500 ng/ml Hoechst 33342 trihydrochloride trihydrate (Invitrogen). Samples were mounted with ProLong Diamond Antifade mountant (Invitrogen) and were imaged with a Nikon 1A confocal microscope (Plan Apo OIL 60× oil, 1.40NA).

3.8. Karyostat and cell ID analysis

The genomic DNA of Ctrl and PLN-R9C iPSCs was analyzed using the KaryoStat™ assay and Cell ID assay (Thermo Fisher), which were based on 150 k SNP probes across the human genome and allowed high-resolution chromosome karyotyping and DNA fingerprint matching of human cell lines.

3.9. Mycoplasma and pX459 vector backbone PCR

PCR Mycoplasma Test Kit I/C (PromoCell GmbH) was used to confirm the absence of mycoplasma according to manufacturer’s instructions. To detect potential genome integration of pX459 vector, the genomic DNA of both Ctrl and PLN-R9C iPSCs, as well as the diluted pX459 plasmid controls were amplified with plasmid backbone primer sets (Table 2). PCR products were examined by agarose gel electrophoresis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Generation and characterization of the PLN-R9C iPSC line using CRISPR/Cas9.
## Table 1

| Classification (Optional italicized) | Test                                                                 | Result                                                                 | Data |
|-------------------------------------|----------------------------------------------------------------------|------------------------------------------------------------------------|------|
| **Morphology**                      | Photography                                                          | Typical iPSC morphology                                                | Fig. 1 panel C |
| Pluripotency status evidence for the described cell line | Qualitative analysis: Immunofluorescence staining | Positive for pluripotency markers: Oct4, SOX2, NANOG, and SSEA4 | Fig. 1 panel D |
|                                     | Quantitative analysis: Flow cytometry Real-time PCR                  | Cell surface markers: 98% of cells are positive for Tra-1–60 in FACS analysis. SOX2, OCT4, and NANOG gene expression in PLN-R9C iPSCs were 351 ± 6, 3902 ± 83, and 167 ± 3 fold higher compared to PLN-R9C iCM | Fig. 1 panel E and F |
| **Karyotype**                       | Whole genome array: KaryoStat™ assay with a resolution of 1–2 Mb     | Normal karyotype: 46 XY for UPITI002-A-1                                 | Fig. 1 panel G |
| Genotyping for the desired genomic alteration | PCR and Sanger sequencing across the edited site                   | Heterozygous PLN NM_002667.5: c.25C > T                               | Fig. 1 panel B |
| Verification of the absence of random plasmid integration events | PCR                                                                 | Negative PCR detection of plasmid backbone sequences in the genomic DNA | Supplementary Fig. 1 panel B |
| Parental and modified cell line genetic identity evidence | Whole genome array-based identification of 150 k genetic SNPs       | Cell ID report showed a high correlation (99.34%) between Ctrl and PLN-R9C iPSCs | Fig. 1 Panel H |
| Mutagenesis/genetic modification outcome analysis | Sequencing of genomic DNA PCR product [mandatory]                  | Confirmed the precise introduction of Heterozygous PLN NM_002667.5: c.25C > T | Fig. 1 panel B |
| Off-target nuclease analysis        | PCR and Sanger sequencing across top 5 predicted off-target sites   | The most likely off-targets were not changed after CRISPR/Cas9 modification | Supplementary Fig. 1 Panel C and D (Predicted off-target sites and Sanger sequencing panels) |
| Specific pathogen-free status       | Mycoplasma [mandatory]                                               | Mycoplasma testing by mycoplasma PCR detection kit assay                | Supplementary Fig. 1 panel A |
| Multilineage differentiation potential | Trilineage in vitro differentiation and immunofluorescence analysis | Positive for three germ layer-specific markers: TUBB3, BRACYHURY, and SOX17 | Fig. 1 I |
| 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   |
### Table 2

Reagents details.

| Antibodies and stains used for immunocytochemistry/flow-cytometry | Dilution | Company Cat # and RRID |
|---------------------------------------------------------------|----------|------------------------|
| **Pluripotency marker**                                      |          |                        |
| OCT4A (C30A3) Rabbit mAb                                     | 1:400    | Cell Signaling Technology Cat#2840S RRID: AB_2167691 |
| SOX2 (L1D6A2) Mouse mAb                                      | 1:400    | Cell Signaling Technology Cat#4900S RRID: AB_10560516 |
| NANOG (D73G4) XP Rabbit mAb                                  | 1:200    | Cell Signaling Technology Cat#4903S RRID: AB_10559205 |
| SSEA (MC813) Mouse mAb                                       | 1:500    | Cell Signaling Technology Cat#755S RRID: AB_1264259 |
| TRA-1–60(S) Mouse mAb                                        | 1:400    | Cell Signaling Technology Cat#746S RRID: AB_2119059 |
| **Ectoderm marker**                                          |          |                        |
| β3-Tubulin (D71G9) XP Rabbit mAb                             | 1:400    | Cell Signaling Technology Cat#5568S RRID: AB_16694505 |
| **Endoderm marker**                                          |          |                        |
| SOX17 (D1T8M) Rabbit mAb                                     | 1:3200   | Cell Signaling Technology Cat#81778S RRID: AB_2650582 |
| **Mesoderm marker**                                          |          |                        |
| Brachyury (D2Z3J) Rabbit mAb                                 | 1:1600   | Cell Signaling Technology Cat#81694S RRID: AB_2799983 |
| **Secondary antibody**                                       |          |                        |
| AlexaFluor488 Goat anti-Rabbit IgG (H + L)                   | 1:2000   | Invitrogen Cat#A11008 RRID: AB_143165 |
| AlexaFluor594 Goat anti-Rabbit IgG (H + L)                   | 1:2000   | Invitrogen Cat#A11012 RRID: AB_141359 |
| AlexaFluor488 Goat anti-Mouse IgG (H + L)                    | 1:2000   | Invitrogen Cat#A11001 RRID: AB_2234609 |
| AlexaFluor594 Goat anti-Mouse IgG (H + L)                    | 1:2000   | Invitrogen Cat#A11005 RRID: AB_2234073 |
| **Nuclear stain**                                            |          |                        |
| Hoechst 33342 trihydrochloride trihydrate                    | 1:20000 (0.5 μg/ml) | Invitrogen Cat#H3570 |
| **Primers and Oligonucleotides used in this study**         |          |                        |
| Target                                                       | Forward/Reverse primer (5’–3’) |
| Genotyping of PLN mutation and genome-editing                |          |                        |
| Genomic DNA amplification                                    | PLN      | CACAAATGAGACGTTGTCATCG  |
|                                                             |          | TTTGTGAGCCATGTTGAGGA    |
| Genomic DNA sequencing                                       | PLN      | TAAGCTGATGGCAAGGCTG     |
### Real-time PCR quantification of mRNA expression

| Housekeeping gene (RT-qPCR) | GAPDH | GGAGCGGAGATCCTCCAAAAT | GGCTGTGTCATACCTCTCAGG |
|-----------------------------|-------|------------------------|------------------------|
| Pluripotency marker (RT-qPCR) | NANOG | TGAACCTCAGCTACAAACAG | TGATGAGGAAGAGTAAAG |
| Pluripotency marker (RT-qPCR) | OCT4  | CCTCACTTCATGCACCTGTA | CAGGGTTTCTTTCTAGCT |
| Pluripotency marker (RT-qPCR) | SOX2  | CCCAGCAGACTTCACATGT | CCTCCCCCCTCCTT |

### Genomic integration of CRISPR vector

| Plasmid Backbone pX459 (PCR) | pac | CCAATGACCGAGTACAAGC GCTCGTAGAAGGGGAGGTT |
|-----------------------------|-----|---------------------------------------------|
| Plasmid Backbone pX459 (PCR) | ampR/ori | TTGCGGATTTCGGTCTATT AGCAAAAACAGGAAGC |

### sgRNA and ssODN designs

- **sgRNA oligos**
  - PLN
  - CACCGTTGAGGCATTTCAATGGTTG AGAAGAATGGCATGGAGAC

- **single-stranded oligo donor (ssODN)**
  - PLN-R9C mutation introduction
  - TCTCTCGACACCTTTAAACTTCAGACCTCTCTGTGC |

### Potential sgRNA off-target sites PCR

| Off-target 1 (PCR) | Chr12. 10075479 | AGAGCAGCGAGCCCTATTTT AGATGAAATGGCATGGAGAC |
|-------------------|-----------------|---------------------------------------------|
| Off-target 2 (PCR) | Chr17. 34095286 | AGGCAGAGAATTTGTGAGA GGTGAGACAGAGATGAGAG |
| Off-target 3 (PCR) | Chr2. 19800777 | CAAAGGGACATGAAGGAC TCCCTCCCTGTAAGAAC |
| Off-target 4 (PCR) | Chr7. 27327363 | GATGACGAGCTCTCTCTGTGACATGGTAGTAAGT |
| Off-target 5 (PCR) | Chr21. 24668849 | TTGGCTGACCCATTAAACTC GGAGCAGGAGATGAGAG |

### Sequencing of off-target PCR products in plasmid (pMiniT 2.0 vector)

- **Upstream primer**
  - ACCTGCAACCAAGCGAGAAC |
- **Downstream primer**
  - TCAAGGTTATGTCATGAGCG
| **Resource Table:** |
|---------------------|
| **Unique stem cell line identifier** | UPITTi002-A-1 |
| **Alternative name(s) of stem cell line** | PLN-R9C |
| **Institution** | Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA, U.S. |
| **Contact information of the reported cell line distributor** | Haodi Wu, haodi@pitt.edu |
| **Type of cell line** | iPSC |
| **Origin** | Human |
| **Additional origin info (applicable for human ESC or iPSC)** | Age: 45 |
| | Sex: male |
| **Cell Source** | Blood |
| **Method of reprogramming** | Integration-free Sendai virus expressing human OCT4, SOX2, KLF4, and c-MYC |
| **Clonality** | Clonal |
| **Evidence of the reprogramming transgene loss (including genomic copy if applicable)** | N/A |
| **Cell culture system used** | N/A |
| **Type of Genetic Modification** | Induced mutation |
| **Associated disease** | Dilated Cardiomyopathy (DCM) |
| **Gene/locus** | PLNChr.6q22.31, Heterozygous PLNNM_002667.5:c.25C > T |
| **Method of modification/site-specific nuclease used** | CRISPR/Cas9 |
| **Site-specific nuclease (SSN) delivery method** | Plasmid transfection |
| **All genetic material introduced into the cells** | sgRNA and Cas9 plasmid, single-stranded oligo donor (ssODN) |
| **Analysis of the nuclease-targeted allele status** | Targeted PCR/sequencing |
| **Method of the off-target nuclease activity surveillance** | Targeted PCR/sequencing |
| **Name of transgene** | N/A |
| **Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)** | Puromycin |
| **Inducible/constitutive system details** | N/A |
| **Date archived/stock date** | UPITTi002-A-1: 03/15/2021 |
| **Cell line repository/bank** | https://hpscreg.eu/cell-line/UPITTi002-A-1 |
| **Ethical/GMO work approvals** | The generation of genome-edited iPSC lines was approved by the Institutional Biosafety Committee (IBC) at the University of Pittsburgh under IBC# 202,000,147 for working with human iPSC cell lines, genome editing, and using recombinant DNAs. |
| **Addgene/public access repository recombinant DNA sources’ disclaimers (if applicable)** | N/A |