Generation of two iPSC lines from hypertrophic cardiomyopathy patients carrying MYBPC3 and PRKAG2 variants

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Abstract

Hypertrophic cardiomyopathy (HCM) is an inherited cardiac disorder characterized by a thick left ventricular wall and an increased risk of arrhythmias, heart failure, and sudden cardiac death. The \textit{MYBPC3} and \textit{PRKAG2} are known causal genes for HCM. Here we generated two human-induced pluripotent stem cell lines from two HCM patients carrying two heterozygous mutations in \textit{MYBPC3} (c.459delC) and \textit{PRKAG2} (c.1703C > T). Both iPSC lines expressed pluripotent markers, had a normal karyotype, and were able to differentiate into three germ layers, making them potentially valuable tools for modeling HCM \textit{in vitro} and investigating the pathological mechanisms related to these \textit{two} variants.

Keywords

Hypertrophic cardiomyopathy; Induced pluripotent stem cells; MYBPC3; PRKAG2

1. Resource table

| Unique stem cell lines identifier | SCVIi036-A, SCVIi037-A |
|----------------------------------|-------------------------|
| Institution                      | Stanford Cardiovascular Institute |
| Contact information of the reported cell line distributor | Dr. Joseph C. Wu; joewu@stanford.edu |
| Type of cell lines               | iPSC |
| Origin                           | Human |
| Additional origin info           | SCVIi036-A: Age:30, Sex: Male; Ethnicity: Southeast Asian  
2. SCVIi037-A: Age:31, Sex: Male; Ethnicity: Southeast Asian |
| Cell Source                      | PBMCs |

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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.
2. Resource utility

Patients carrying these two mutations, MYBPC3 (c.459delC) and PRKAG2 (c.1703C > T), showed a hypertrophic cardiomyopathy phenotype. The iPSC lines generated from these patients provide a source to differentiate into cardiomyocytes under in vitro conditions and can be used to study the pathophysiology of the disease by serving as a potential tool for drug screening.

3. Resource details

Hypertrophic cardiomyopathy (HCM) is an inherited autosomal dominant disorder characterized by intraventricular septum and left ventricular wall thickening leading to diastolic dysfunction as well as an increased risk of ventricular and atrial arrhythmias. HCM is one of the leading causes of sudden cardiac death in the US. HCM has a heterogeneous clinical presentation with symptoms ranging from asymptomatic to mild to critical heart failure (Marian and Braunwald, 2017; Lan et al., 2013). Irrespective of age and sex, HCM affects more patients than any other form of cardiomyopathy. HCM is linked to mutations in multiple genes involved in the generation of heart muscle proteins, such as β myosin heavy chain (β-MHC) and myosin-binding protein C (MYBPC3), in the energy demand regulation by AMP-activated protein kinase subunit gamma-2 (PRKAG2), and others.

Although some mutations in canonical HCM genes are definitely causal for the disease phenotype, in other cases, it can be difficult to determine if the mutation that is identified on genetic testing is causal for disease or an “innocent bystander”. These variants are referred to as “variants of uncertain significance”, and better methods and model systems to determine the pathogenicity of these variants are critical for patient care and for understanding biology.

MYBPC3 is responsible for maintaining a cardiac contraction, but its frameshift variant (c.459delC) has a direct pathogenic association with a structural component of the cardiomyocytes (Seeger et al., 2019). PRKAG2 is responsible for regulating cardiomyocyte...
energetics and known pathologic variants have been identified in HCM. Currently, the missense variant (c.1703C > T) is annotated as a variant of uncertain significance (VUS). Other PRAKG2 mutations are predicted to cause Wolff-Parkinson-White syndrome, ventricular hypertrophy, and conduction system disease (Porto et al., 2016).

With the help of patient-specific induced pluripotent stem cells (iPSCs), we can quickly develop an in vitro screening platform to mimic HCM disease phenotype with MYBPC3 (c.459delC, pathogenic) and PRAKG2 (c.1703C > T, VUS). Furthermore, these iPSCs can be differentiated into cardiomyocytes, thus providing us with a tool to conduct a relevant clinical trial in a dish.

Here, we generated iPSCs lines from a 30-year old (SCVIi036-A) and a 31-year old (SCVIi037-A) male, both individuals carrying MYBPC3 (c.459delC, coding for p.Ile154LeufsX5) and PRAKG2 (c.1703C > T, coding for p.Thr568Met) variants (Resource Table). Both patients’ peripheral blood mononuclear cells (PBMCs) were reprogrammed to iPSCs using the Sendai virus vector containing Oct3/4, Sox2, Klf4, and c-Myc (Yamanaka factors). These iPSC clones showed typical morphology. The scale bar is 370 μm (Fig. 1A). The iPSC lines from these patients showed the expression of pluripotency markers OCT3/4, NANOG, and SOX2 as detected by immunostaining. The scale bar is 130 μm (Fig. 1B). These iPSC lines were able to differentiate into the three germ layers: endoderm, mesoderm, and ectoderm. The scale bar is 130 μm (Fig. 1C). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) confirmed the presence of NANOG and SOX2 at mRNA level in both lines compared to the positive control iPSC line SCVI15 (Fig. 1D). Further, we established the loss of the Sendai virus vector for these two lines (Fig. 1E). MYBPC3-specific (c.459delC) and PRKAG2-specific (c.1703C > T) heterozygous mutations were confirmed by Sanger sequencing as compared to the control iPSC line SCVI15 (Fig. 1F). All of the lines had a normal karyotype as assessed by the KaryoStat assay (Fig. 1G) and were found to be free from mycoplasma contamination. Short tandem repeat (STR) analysis proved that the genetic origin of these iPSC lines is the same as of their donor PBMCs (Submitted in the archive with journal).

4. Materials and methods

4.1. Reprogramming

PBMCs were isolated from blood using Percoll density gradient medium (#17089109, GE Healthcare), purified using DPBS, and plated in a 24-well plate as previously described (Belbachir et al., 2021). Cells were cultured in StemPro®-34 SFM medium (#10639011, Thermo Fisher Scientific) and nourished with specific supplements: SCF (100 ng/mL, #300–07, Peprotech), FLT3 (100 ng/mL, #PHC9414, Thermo Fisher Scientific), IL-3 (20 ng/mL, #200–3, Peprotech), IL-6 (20 ng/mL, #PHC0063, ThermoFisher Scientific), and EPO (20 ng/mL, #PHC9631, Thermo Fisher Scientific). PBMCs were reprogrammed according to the instructions provided with CytoTune™-iPSC 2.0 Sendai Reprogramming Kit (#A16517, Thermo Fisher Scientific). Transduced PBMCs were resuspended and plated on a Matrigel-coated plate. Cells were cultured in StemPro™-34 medium (Thermo Fisher Scientific).

On day 7, the medium was redirected to StemMACSTM iPSC-Brew XF medium (#130–104–
368, Miltenyi Biotec), and cells were maintained until days 10–15 post-transduction. Cell colonies were picked, and clones expanded as previously described (Belbachir et al., 2021).

4.2. Cell culture

iPSCs were cultured in sterile conditions using StemMACS iP-S-Brew XF medium in a humidified incubator with 5% CO$_2$ at 37 °C. A concentration of 10 μM of ROCK inhibitor (#Y27632, Selleck Chemicals) was used for the first 24 h only after every passaging. Cells were replenished with fresh medium every other day and observed until confluency.

4.3. Trilineage differentiation

To validate the inherent property of both lines, iPSCs were differentiated at passage 13 into three germ layers—endoderm, mesoderm, and ectoderm protocol (#05230, STEMCELL™ Technologies).

4.4. Immunofluorescence

The iPSCs or iPSC-differentiated germ layers (endoderm, mesoderm, and ectoderm) were fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilized with 50 μg/mL digitonin (#D141, Sigma-Aldrich) for 10 min and subsequently blocked with 1% Bovine Serum Albumin (#A7030, SigmaAldrich) and 5% serum (Donkey Serum, #D9663, Sigma-Aldrich; Goat Serum, #31873, Thermo Fisher Scientific) at room temperature. After overnight incubation with specific primary antibodies (Table 2) at 4°C, cells were washed and incubated with their particular secondary antibodies (Table 2) for 30 min at room temperature. Cell nuclei were stained with the NucBlue (#R37606, Thermo Fisher Scientific) and images captured.

4.5. RT-PCR

2 × 10$^6$ iPSCs from each line at passage 12 were collected in TRIzol® and processed for total RNA extraction using Direct-zol™ RNA Miniprep Kit (#R2050, Zymo Research) according to the instructions provided in the manufacturer’s protocol. Following cDNA synthesis by using iScript™cDNA Synthesis Kit (#1708891, BioRad), RT-PCR was performed to evaluate the expression of NANOG, SOX2, SEV, and SEV-KOS using primers (Table 2) and TaqMan™ Gene Expression Assay (#4444556, Applied Biosystems™).

4.6. Karyotyping

2 × 10$^6$ iPSCs from each line were collected at passage 11 and processed for a whole-genome array to detect chromosomal abnormality by using the KaryoStat™ assay (Thermo Fisher Scientific).

4.7. Short tandem repeat analysis

To validate the origin of iPSCs lines, genomic DNA from PBMCs and iPSCs were isolated and purified by using DNeasy Blood & Tissue Kit (#69504, Qiagen). Further, CLA IdentiFiler™ Direct PCR Amplification Kit (#A44660, Thermo Fisher Scientific) was used, and amplified products were analyzed using capillary electrophoresis on ABI3130xl by the Stanford Protein Nucleic Acid (PAN) Facility.
4.8. Sequencing

To evaluate the genomic region of interest, extracted genomic DNA from iPSC lines by using DNeasy Blood & Tissue Kit (#69504, Qiagen) and performed a PCR reactions with primers (Table 2) targeting the region of interest by using the HighFidelity kit (#M0541S, New England Biolabs). PCR products were purified using the QIAquick Purification Kit (#28706, Qiagen) and subjected to sequence analysis on ABI3130xl by the Stanford PAN facility.

4.9. Mycoplasma detection

Mycoplasma contamination was assessed using the MycoAlert Detection Kit (#LT07–118, Lonza) as per the manufacturer’s protocol.

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Fig. 1.
Characterization of iPSC lines derived from hypertrophic cardiomyopathy patients carrying MYBPC3 (c.459delC) and PRKAG2 (c.1703C>T) mutations.
### Table 1

| Classification     | Test                                                      | Result                                                                 | Data                      |
|--------------------|-----------------------------------------------------------|------------------------------------------------------------------------|---------------------------|
| Morphology         | Photography bright field                                   | Normal                                                                 | Figure 1A                 |
| Phenotype          | Qualitative analysis (Immunocytochemistry)                 | Positive expression of pluripotency markers: Oct3/4, NANOG, SOX2       | Figure 1B                 |
| Genotype           | Quantitative analysis (RT-qPCR)                            | mRNA expression of SOX2 and NANOG                                      | Figure 1D                 |
|                    | Karyotype: Whole genome array (KaryoStat™ Assay)           | Normal karyotype: 46 XY for SCVIi036-A                                  | Figure 1G                 |
|                    | Resolution 1–2 Mb                                          | XY for SCVIi037-A                                                      |                           |
| Identity           | Microsatellite PCR (mPCR) or STR analysis                  | N/A                                                                    | N/A                       |
| Mutation analysis  | Sequencing                                                | Heterozygous MYBPC3 (c.459delC) Heterozygous PRKAG2 (c.1703C > T)     | Figure 1F                 |
| Microbiology and virology | Mycoplasma                                              | Luminescence: Negative                                                |                           |
| Differentiation potential | Directed differentiation, Immunofluorescence staining for 2 markers per germ layer | Positive Immunofluorescence staining of three germ layer markers: Ectoderm: PAX6, OTX2, Endoderm: SOX17, FOXA2, Mesoderm: BRACHYURY, TBX6 | Figure 1C                 |
| Donor screening    | HIV 1 + 2, Hepatitis B, Hepatitis C                        | N/A                                                                    | N/A                       |
| Genotype additional info | Blood group genotyping                                    | N/A                                                                    | N/A                       |
|                    | HLA tissue typing                                         | N/A                                                                    | N/A                       |
### Table 2

Reagents details.

| Antibodies used for Immunocytochemistry | Antibody | Dilution | Company Cat # | RRID  |
|----------------------------------------|----------|----------|---------------|-------|
| Pluripotency Markers                   | Rabbit Anti-NANOG | 1:200 | Proteintech Cat# 142951-1-AP | AB_1607719 |
|                                        | Mouse IgG2κ Anti-OCT-3/4 | 1:200 | Santa Cruz Biotechnology Cat# sc-5279 | AB_628051 |
|                                        | Mouse IgG1κ Anti-SOX2 | 1:200 | Santa Cruz Biotechnology Cat# sc-365823 | AB_10842165 |
| Ectoderm Markers                       | Goat Anti-OTX2 | 1:200 | R&D Systems Cat# 963,273 | AB_2157172 |
|                                        | Rabbit Anti-Pax6 | 1:100 | Thermo Fisher Scientific Cat# 42-6600 | AB_2533534 |
| Endoderm Markers                       | Goat Anti-SOX17 | 1:200 | R&D Systems Cat# 963,121 | AB_355060 |
|                                        | Rabbit Anti-Foxa2 | 1:250 | Thermo Fischer Scientific Cat# 701,698 | AB_2576439 |
| Mesoderm Markers                       | Goat Anti-Brachyury | 1:200 | R&D Systems Cat# 963,427 | AB_2200235 |
| Secondary Antibodies                   | Alexa Fluor 488 Goat Anti-Mouse IgG1 | 1:1000 | Thermo Fisher Scientific #A-21121 | AB_2535764 |
|                                        | Alexa Fluor 488 Donkey Anti-Goat IgG (H + L) | 1:1000 | Thermo Fisher Scientific #A-11055 | AB_2534102 |
|                                        | Alexa Fluor 555 Goat Anti-Rabbit IgG (H + L) | 1:500 | Thermo Fisher Scientific #A-21428 | AB_141784 |
|                                        | Alexa Fluor 647 Goat Anti-Mouse IgG2b | 1:250 | Thermo Fisher Scientific #A-21242 | AB_2535811 |

| Primers                               | Target | Forward/Reverse primer (5’-3’) |
|----------------------------------------|--------|-------------------------------|
| Sendai Virus (qPCR)                    | Sendai Virus genome | Mr04269880_mr |
|                                        | Sendai-KLF4-KOS | Mr04421257_MR |
| Genotyping                             | MYBPC3 (c.459delC) | Fwd: GCTCTCAATGGTCCTACCCC, Rev: TCTCTCCGTGTCCTCCACGAC |
|                                        | PRKAG2 (c.1703C > T) | Fwd: ACATACCGTGACTCACCCT, Rev: TACGTGGATCCAAGCTGC |
| House-Keeping Gene (qPCR)              | GAPDH | HS02786624_g1 |
|                                        | 18s   | HS03003631_g1 |
|                                        | SOX2  | HS04234836_s1 |
|                                        | NANOG | HS02387400_g1 |