Phenotypic Screening Using Patient-Derived Induced Pluripotent Stem Cells Identified Pyr3 as a Candidate Compound for the Treatment of Infantile Hypertrophic Cardiomyopathy

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Summary

Hypertrophic cardiomyopathy (HCM) is a genetic disorder characterized by hypertrophy of the myocardium. Some of the patients are diagnosed for HCM during infancy, and the prognosis of infantile HCM is worse than general HCM. Nevertheless, pathophysiology of infantile HCM is less investigated and remains largely unknown. In the present study, we generated induced pluripotent stem cells (iPSCs) from two patients with infantile HCM: one with Noonan syndrome and the other with idiopathic HCM. We found that iPSC-derived cardiomyocytes (iPSC-CMs) from idiopathic HCM patient were significantly larger and showed higher diastolic intracellular calcium concentration compared with the iPSC-CMs from healthy subject. Unlike iPSC-CMs from the adult/adolescent HCM patient, arrhythmia was not observed as a disease-related phenotype in iPSC-CMs from idiopathic infantile HCM patient. Phenotypic screening revealed that Pyr3, a transient receptor potential channel 3 channel inhibitor, decreased both the cell size and diastolic intracellular calcium concentration in iPSC-CMs from both Noonan syndrome and idiopathic infantile HCM patients, suggesting that the target of Pyr3 may play a role in the pathogenesis of infantile HCM, regardless of the etiology. Further research may unveil the possibility of Pyr3 or its derivatives in the treatment of infantile HCM.

Key words: Patient-specific iPSCs, High-content imaging, Calcium imaging, Transient receptor potential channel inhibitor

Hypertrophic cardiomyopathy (HCM) is a genetic disorder characterized by left ventricular hypertrophy with histopathological findings of cardiomyocyte enlargement, disarray, and interstitial fibrosis.6,7 The prevalence of HCM has been reported as 1 in 500 in general population.2 The prognosis of HCM is generally good, especially for those who are diagnosed later in life. Mutations in genes encoding contractile myofilaments of sarcomere or adjacent Z-disc have been identified in more than half of the patients with HCM,6 and hypersensitivity of the myofilament contraction against calcium that is induced by gene mutation has been shown as the fundamental mechanism underlying the pathophysiology of HCM.4,5 Most of the patients with HCM develop the symptoms and are diagnosed at adolescents or adults, but some of the patients develop HCM and are diagnosed at infancies. The etiologies of these “infantile HCM” include syndromic HCM caused by inborn errors of metabolism, muscular dystrophy, and malformation syndromes in addition to idiopathic HCM. Although the gene mutation that causes idiopathic HCM in infants is shared with adults/adolescents,6 the prognosis of these infantile HCM, especially those diagnosed before 1-year-old, is worse compared with general HCM.7 Nevertheless, pathophysiology of the infantile HCM, including the similarity and difference between general HCM, still remains largely unknown, and investigation of the mechanism and therapeutic target of infantile HCM is important for improving the prognosis of
the patients with infantile HCM.

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Human induced pluripotent stem cells (iPSCs) are the pluripotent stem cells that could be generated from any individuals and are expected to be useful for many purposes including drug discovery for genetic diseases. Establishment of iPSCs from HCM patients and characterization of iPSC-derived cardiomyocytes (iPSC-CMs) from HCM patients (HCM iPSC-CMs), including those with malformation syndrome, have already been reported from several investigators. For example, HCM iPSC-CMs carrying mutation in sarcomere gene, MYH7, are reported to recapitulate the expected disease-specific phenotypes, namely cellular hypertrophy, abnormal Ca\(^{2+}\) transient, and liability to arrhythmia. Importantly, at least some of the disease-specific phenotypes could be normalized by current therapeutics against HCM, such as \(\beta\)-blocker and calcium channel antagonists, suggesting that HCM iPSC-CMs would be useful for drug discovery.

In the present study, we established iPSCs from two patients with HCM who were diagnosed before birth: one with Noonan syndrome and the other with idiopathic HCM. We found that iPSC-CMs of idiopathic HCM patient exhibit disease-specific phenotypes, namely cellular hypertrophy, impaired calcium homeostasis, and hypersensitivity against \(\beta\)-adrenergic stimulation. We then performed a phenotypic drug screening that could normalize these disease-specific phenotypes and identified Pyr3, an inhibitor of transient receptor potential channel 3 (TRPC3).

**Methods**

**Establishment of iPSCs:** We established iPSCs from T cells essentially as described previously. We used mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan)-inactivated mouse embryonic fibroblasts as a feeder layer and medium consisting of Knockout DMEM/F12 supplemented with 20% Knockout serum replacement (KSR), 100 \(\mu\)M nonessential amino acids, 1 × GlutaMAX, 50 U/mL penicillin, 50 \(\mu\)g/mL streptomycin, 0.1 mM \(\beta\)-mercaptoethanol (Thermo Fisher Scientific, Tokyo, Japan), and 10 ng/mL bFGF (WAKO, Osaka, Japan). The medium was changed every other day. Quality of the iPSCs was verified by the expression of pluripotency factors and teratoma formation in nude mice. We also used 253G1 iPSCs (RIKEN BioResource Center) and iPSCs established from healthy volunteers (HCO1-8) as controls. Characterization of the established iPSCs was performed as described previously.

Written informed consent was obtained from the donor or their guardians under the protocol approved by the Institutional Review Board of Osaka University (Approval number 13254(829-1)-3) and The University of Tokyo (G 10019).

**Cardiac differentiation, purification, and quality analysis of iPSC-CMs:** Differentiation of iPSCs into cardiomyocytes was performed by stage-specific activation and inhibition of Wnt signaling. Sub-confluent iPSCs colonies were dissociated into small clumps to generate embryoid bodies (EBs) in modified StemPro-34 (mST34) medium (Invitrogen, Tokyo, Japan), including 1 × GlutaMAX, 1 mM ascorbic acid, and 0.4 mM monothioglycerol (MTG) (Invitrogen, Tokyo, Japan), 2.5% KSR, and 10 \(\mu\)M Y-27632. Thereafter, EBs were serially treated with CHIR99021 (4.5 \(\mu\)M), a GSK inhibitor that activates Wnt signaling, and IWP2 (5 \(\mu\)M), a porcupine inhibitor that inhibits Wnt signaling, in mST34 without Y-27632 on day 1 and day 5, respectively. We also used TGF-\(\beta\) inhibitors SB431542 (5 \(\mu\)M) and Dorsomorphin (0.5 \(\mu\)M) from day 3 to day 5 to enhance cardiomyocyte production. Purification of cardiomyocytes was performed by culturing the dissociated EBs in a glucose-free medium supplemented with 4 mM lactic acid for 3-5 days, and the culture was continued in DMEM containing 2% FBS until phenotypic analysis. All cultures were maintained in a 37°C, 5% CO\(_2\) condition.

**Immunofluorescent staining:** iPSC-CMs were plated onto Geltrex (Thermo Fisher Scientific, Tokyo, Japan)-coated Lab-Tek\(^{\circ}\) chamber slides (Nunc, Tokyo, Japan) or 96-well black well clear bottom plate (Cell carrier-96, PerkinElmer, Yokohama, Japan). After the culture and drug treatment, iPSC-CMs were fixed with 4% PFA for 15 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with 5% goat serum, 2% bovine serum albumin (BSA) for 1 hour at room temperature, and incubated with primary antibody at 4°C overnight. Primary antibodies were anti-Troponin T (clone 13-11) (1:400, Thermo Scientific, Tokyo, Japan), anti-Titin (1:400, Synaptic Systems, Goettingen, Germany, Cat. # 311 011), and anti-MLC2a (1:400, Biogenesis, Hertfordshire, UK). After washing thrice in PBS, iPSC-CMs were incubated with secondary antibodies (donkey anti-mouse IgG Alexa Fluor\(^{\circ}\) 488, Cat. # R37114, donkey anti-rabbit IgG Alexa Fluor\(^{\circ}\) 594, Cat. # R37119, 1:500, Thermo Fisher Scientific, Tokyo, Japan) for 1 hour at room temperature. Cells on Lab-Tek\(^{\circ}\) chamber slides were counterstained with DAPI and mounted with ProLong\(^{\circ}\) Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, Tokyo, Japan). Cells on 96-well plates were counterstained with NucBlue\(^{\circ}\) (Thermo Fisher Sci-

**Table I. Patient Characteristics**

<table>
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<tr>
<th>Patient</th>
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<th>HCM(^{a/b})</th>
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<td>2</td>
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<tr>
<td>Sex</td>
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<td>Male</td>
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<tr>
<td>Cardiac phenotypes</td>
<td>HCM at birth</td>
<td>HCM at birth</td>
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<tr>
<td>Family history</td>
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<td>None</td>
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<tr>
<td>Other cardiac complications</td>
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<td>Nonsustained ventricular tachycardia</td>
</tr>
<tr>
<td>Gene mutation</td>
<td>770C&gt;T in exon 7 of RAF1 gene</td>
<td></td>
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</table>
Figure 1. Hypertrophy in patient-derived iPSC-CMs. A: Representative images of the iPSC-CMs from control (Ctrl) and iHCM at days 30, 45, and 60 of differentiation. iPSC-CMs were stained with cardiac Troponin T (cTnT) and DAPI. Scale bar, 100 μm. B: Quantification of the cell area. The cell area of iPSC-CMs from control (Ctrl), HCM-RAFF1, and iHCM at days 30, 45, and 60 of differentiation was analyzed by high-content imaging system. Each blue circle (control (Ctrl), n = 20 per each time point), green reverse triangle (HCM-RAFF1, n = 15 per each time point), and red square (iHCM, n = 20 per each time point) represents the mean cell area of iPSC-CMs in 1 well of 96-well plate. C: Representative images of the iPSC-CMs from control (Ctrl) and iHCM at day 60 of differentiation. iPSC-CMs were stained with MLC2a, MLC2v, and DAPI. Scale bar, 100 μm. D: The percentage of MLC2a+/MLC2v-, MLC2a+/MLC2v+, and MLC2a-/MLC2v+ cells in control and iHCM iPSC-CMs was analyzed by high-content imaging system (n = 3 each). Ctrl, control. *P < 0.05, **P < 0.01, ****P < 0.0001; N.S, not significant in two-way ANOVA followed by Tukey’s post hoc test. The bar shows the mean, and the error bar represents SD.

Scientific, Tokyo, Japan) and CellMask™ Deep Red stain (Thermo Fisher Scientific, Tokyo, Japan). Fluorescent images were acquired by LSM700 confocal microscope (Zeiss, Tokyo, Japan) or Operetta® high content imaging system (PerkinElmer, Yokohama, Japan) and analyzed using Columbus™ image data storage and analysis system (PerkinElmer, Yokohama, Japan) and Harmony® analysis software (PerkinElmer, Yokohama, Japan).

Calcium imaging and absolute quantification of intracellular calcium concentration: iPSC-CMs were plated onto Geltrex® (Thermo Fisher Scientific, Tokyo, Japan)-coated 96-well black well clear bottom plate (Corning, Tokyo, Japan) at 25,000 cells per well. On day 7, iPSC-CMs were washed with FluoroBrite DMEM (Thermo Fisher Scientific, Tokyo, Japan) supplemented with 1 × GlutaMAX™ and 10 mM HEPES, and then stained with 5 μM Indo-1 AM (Thermo Fisher Scientific, Tokyo, Japan) with 0.1% Pluronic F-127 (Thermo Fisher Scientific,
After 5 days, the cells were fixed and stained with anti-dium and the compounds were refreshed every other day. concentrations. DMSO was used as a solvent control. Me-
cells were treated with various compounds at the indicated calcium imaging, respectively. Two days after the plating, 25,000 cells per well for high-content imaging and cal-
coated 96-well black well clear bottom plate at 3,000 and ₋

Figure 2. Impaired calcium homeostasis in iHCM iPSC-CMs. A: Representative image of the Ca\textsuperscript{2+} transient in spontaneously beating iPSC-CMs from control (Ctrl) and iHCM. Blue line, Ctrl iPSC-CMs; Red line, iHCM iPSC-CMs. B, C: Mean (B) and coefficient of variation (CV, C) of the frequency of spontaneous contraction in iPSC-CMs, analyzed by Ca\textsuperscript{2+} transient (n = 6 each). Ctrl, control. D: Representative image of the Ca\textsuperscript{2+} transient in iPSC-CMs from control (Ctrl) and iHCM, electrolytically stimulated at 1 Hz. Blue, Ctrl iPSC-CMs; Red, iHCM iPSC-CMs. E, F: Analysis of the Ca\textsuperscript{2+} transient in electrolytically stimulated iPSC-CMs at 1 Hz. Time to peak (E) and transient amplitude (F) were analyzed from the Ca\textsuperscript{2+} transient of iPSC-CMs (n = 6 each). Ctrl, control. G: Quantification of absolute diastolic [Ca\textsuperscript{2+}], in iPSC-CMs from control (Ctrl), HCM\textsuperscript{RAFT}, and iHCM. *P < 0.05, **P < 0.01; N.S. indicates not significant in two-tailed t-test or one-way ANOVA followed by Tukey’s post hoc test. The bar shows the mean and the error bar represents SD.

Tokyo, Japan) for 60 minutes at 37°C. Calcium imaging was performed by FDSS/μCELL equipped with 96-
channel multi-electrode array (Hamamatsu Photonics, Hamamatsu, Japan). Fluorescent signals of the free calcium were calculated by the ratio of fluorescent intensity at 409 nm and 495 nm. All experiments were performed at 37°C under atmospheric condition. Calcium transients from iPSC-CMs were averaged for 30 secs and analyzed using MATLAB (Mathworks, Natick, MA) and OriginPro 8.6 (LightStone Mathworks). Quantification of absolute intracellular calcium concentration was performed using Calcium Calibration Buffer Kits (Thermo Fisher Scientific, Tokyo, Japan). Briefly, using the fluorescent signals of Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-containing solutions, calcium concentration was determined according to the following formula:

\[
[Ca^{2+}]_{free} = \frac{K_{dEGTA} \times \text{CaEGTA}}{K_{EGTA}}
\]

Calcium concentration of the iPSC-CMs was determined according to the double log plot.

Phenotypic screening and hit identification: For phenot-
typic screening, iPSC-CMs were plated on Gelrex\textsuperscript{®}
-coated 96-well black well clear bottom plate at 3,000 and 25,000 cells per well for high-content imaging and calcium imaging, respectively. Two days after the plating, cells were treated with various compounds at the indicated concentrations. DMSO was used as a solvent control. Medium and the compounds were refreshed every other day. After 5 days, the cells were fixed and stained with anti-
Troponin T, Hoechst, and HCS CellMask\textsuperscript{TM} Deep Red Stain for high-content screening, or with Indo-1 for calcium imaging. Experiments were repeated in triplicate using three different batches of iPSC-CMs from three independent series of cardiac differentiations.

Statistical analysis: All data were presented as mean ± standard deviation (SD). Indicated sample sizes (n) represent biological replicates from independent series of cardiac differentiation. Statistical significance was determined by using unpaired two-tailed Student’s t-test for two-group comparison or one-way or two-way ANOVA followed by Turkey’s or Dunnett’s post hoc test for analyzing more than two groups. Statistical analyses were done by using GraphPad Prism \textsuperscript{7} software. N.S indicates a nonsignificant difference. P values of < 0.05 were considered statistically significant. * or *, ** or ***, *** or **** indicate P < 0.05, P < 0.01, P < 0.001, and P < 0.0001, respectively.

Results

Generation of iPSCs from patients with infantile HCM: Patient-specific iPSCs were established from two patients (Table I) who were diagnosed as HCM before birth. One of the patient (HCM\textsuperscript{RAFT}) possessed 770C > T mutation in exon 7 of RAF1 gene\textsuperscript{21} and was diagnosed as Noonan syndrome. The other patient (iHCM for idiopathic HCM) showed no signs for inborn errors of metabolism, neuromuscular disease, and malformation syndrome. The pluripotency and the karyotype of iPSCs from both patients was confirmed as previously described (Supplemen-
Hypertrophy in patient-derived iPSC-CMs: iPSCs were differentiated into cardiomyocytes by serial activation and inhibition of Wnt signaling in 3D culture, and iPSC-CMs were purified by metabolic selection. Purity of the iPSC-CMs was approximately 85% in iPSC-CMs from control, iPSC-CMs with positive cardiac troponin T (cTnT) staining was also larger compared with control iPSC-CMs (Figure 1B, green reverse triangle).

Previous studies reported that iPSC-CMs contain several subtypes, namely, atrial, ventricular, and nodal-type iPSC-CMs. To test the possibility that composition of the subtypes affected the phenotype of iHCM iPSC-CMs, we evaluated the subtype of iPSC-CMs by immunostaining with myosin light chain (MLC) 2a and MLC2v, which are preferentially expressed in atrial and ventricular cardiomyocytes, respectively. The cell area of MLC2a+/MLC2v- atrial cardiomyocytes was significantly larger than those of MLC2a+/MLC2v- ventricular cardiomyocytes (Figure 1C). Although the fraction of atrial, relatively small cardiomyocytes tends to be higher in iHCM iPSC-CMs, there were no statistical significance in the composition of cardiomyocyte-subtype be-

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**Figure 3.** Isoproterenol exacerbates disease-specific phenotype of iHCM iPSC-CMs. A: Representative images used for high-content analysis. iPSC-CMs from control (Ctrl) and iHCM were treated with various concentrations of isoproterenol (ISO) and stained with cTnT and Hoechst. Scale bar, 1 mm. B: Representative images of the Ca2+ transient in iPSC-CMs from control (Ctrl) and iHCM, electrically stimulated at 1 Hz. Left panel, Ctrl iPSC-CMs treated with vehicle control (blue) and ISO (orange), Right panel, iHCM iPSC-CMs treated with vehicle control (black) and ISO (red). C: Dose-response curve of ISO-induced hypertrophy. iPSC-CMs from control (Ctrl) (n = 4 for each concentration) and iHCM (n = 4 for each concentration) were treated with various concentrations of ISO, and the cell area was analyzed by high-content imaging system. Y-axis represents the fold-change from the iPSC-CMs treated with vehicle control. The error bar represents SD. D: EC50 was calculated from the dose-response curve in B. The bar shows the mean and the error bar represents 95% confidence interval. E: Dose-response curve of ISO-induced elevation of diastolic [Ca2+]. iPSC-CMs from control (Ctrl) (n = 4 for each concentration) and iHCM (n = 4 for each concentration) were treated with various concentrations of ISO and the levels of diastolic [Ca2+] were quantified. Y-axis represents the percentage of change of diastolic [Ca2+] from the iPSC-CMs treated with vehicle control. The error bar represents SD. F: EC50 was calculated from the dose-response curve in E. The bar shows the mean and the error bar represents 95% confidence interval. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 in one-way ANOVA followed by Tukey’s post hoc test. The bars show the mean and the error bar represents SD.
and plays essential role in cardiac hypertrophy. 23) Altera-
quantified the \([\text{Ca}^{2+}]_i\) in iHCM iPSC-CMs (Figure 2A). 20) iHCM iPSC-CMs, we analyzed the calcium transient and 
investigate whether calcium handling is also impaired in 
CMs electrically stimulated at 1 Hz (Figure 2D).

CMs: We next analyzed the calcium transient of iPSC-
waves in either control, iHCM, or HCM
(CM: [\text{Ca}^{2+}]_i) activates calcium-dependent signaling pathway

Impaired calcium homeostasis in patient-derived iPSC-
iHCM iPSC-CMs at day 50-60 of differentiation.
the disease-specific phenotype became clearer over time,
disease-specific phenotypes of HCM iPSC-CMs.9,10) How-
DADs, respectively) have been reported as one of the
ibilities in calcium handling are also described in mouse
鼠. These results collectively suggest that iPSC-CMs from

Isoproterenol exacerbates disease-specific phenotype of
hCM iPSC-CMs: Patients with HCM have been re-
port to show abnormal response against catechola-
from the lower concentration (Figure 3C-F). These results
iHCM iPSC-CMs (Figure 3A, B). Notably, the effects on

Phenotypic screening using iHCM iPSC-CMs: We next

Table II. List of the Compounds Used for the Phenotypic Screening

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<tr>
<th>Drugs</th>
<th>Targets</th>
<th>Categories</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>1st conc. (µM)</th>
<th>2nd conc. (µM)</th>
<th>3rd conc. (µM)</th>
<th>4th conc. (µM)</th>
<th>5th conc. (µM)</th>
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<td>Irbesartan</td>
<td>GPCR antagonist</td>
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<td>3</td>
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<tr>
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<td>DNA damage response modifier</td>
<td>PARP</td>
<td>20 nM</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Calpain inhibitor I</td>
<td>Protease inhibitor</td>
<td>Calpain</td>
<td>0.09 µM</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

Morphometric analysis revealed that time to peak and the transient amplitude were comparable between control and iHCM iPSC-CMs (Figure 2E, F). Quantification of diastolic [\text{Ca}^{2+}], revealed that diastolic [\text{Ca}^{2+}], was significantly higher in iHCM iPSC-CMs (Figure 2G). The diastolic [\text{Ca}^{2+}], in HCM iPSC-CMs tend to be higher compared with control iPSC-CMs but did not reach statistical significance (Figure 2G). These results collectively suggest that iHCM iPSC-CMs exhibit abnormal calcium handling as a disease-specific phenotype that recapitulates the observations in HCM patients. Isoproterenol exacerbates disease-specific phenotype of iHCM iPSC-CMs: Patients with HCM have been reported to show abnormal response against catecholamines. We, therefore, tested the effect of isoproterenol, a β-adrenergic agonist, on the disease-specific phenotype of iHCM iPSC-CMs at day 50-60 of differentiation.

Impaired calcium homeostasis in patient-derived iPSC-CMs: Elevation of intracellular calcium concentration (\([\text{Ca}^{2+}]_i\)) activates calcium-dependent signaling pathway and plays essential role in cardiac hypertrophy. Alterations in calcium handling are also described in mouse models of cardiomyopathy and in human HCM. To investigate whether calcium handling is also impaired in iHCM iPSC-CMs, we analyzed the calcium transient and quantified the [\text{Ca}^{2+}]_i in iHCM iPSC-CMs (Figure 2A). The frequency of spontaneous contraction (Figure 2B) and its coefficient of variation (Figure 2C) were comparable between control and iHCM iPSC-CMs. Arrhythmia including early or delayed after depolarizations (EADs or DADs, respectively) have been reported as one of the disease-specific phenotypes of HCM iPSC-CMs. However, we could not observe any arrhythmia-like irregular waveforms in either control, iHCM, or HCM iPSC-CMs. We next analyzed the calcium transient of iPSC-CMs electrically stimulated at 1 Hz (Figure 2D).
performed phenotypic screening to investigate the compounds that could normalize the disease-specific phenotypes in iHCM iPSC-CMs. We selected 24 compounds (Table II) that could affect cardiomyocyte hypertrophy, including calcium channel blocker (verapamil) and β-blocker (bisoprolol), which were shown to normalize the disease-specific phenotypes of HCM iPSC-CMs in previous studies.9,10) As cytotoxicity of the compounds may affect the cell size, we first treated iHCM iPSC-CMs with threefold serial dilutions of each compound, starting from 10 μM. Cytotoxicity was assessed by the viability of iHCM iPSC-CMs after the treatment for 5 days. The concentration of each compound with cell viability less than 80% of DMSO (vehicle control)-treated, control iHCM iPSC-CMs were judged as cytotoxic and three- or fivefold serial dilutions at the sub-cytotoxic concentration were used for phenotypic screening.

Cell hypertrophy and elevation of diastolic $[Ca^{2+}]_i$ in iHCM iPSC-CMs are presumed to represent distinct aspect of the disease. We, therefore, tried to search for the compound that would normalize both morphological and physiological disease-specific phenotypes. We used high-content analysis system to evaluate and quantitate the morphological phenotype in a non-biased manner and included isoproterenol and insulin-like growth factor-1 (IGF-1) as a positive control to normalize the results between the assay plate and series of differentiation. After the treatment with compounds for 5 days, we found that 6 compounds, namely Pyr3, rapamycin, bisoprolol, KN-93, cyclosporin A, and PD0325901, decreased the cell area of iHCM iPSC-CMs more than 5% (Figure 4A). On the other hand, only 3 compounds, namely Pyr3, irbesartan, and PD0325901 decreased the diastolic $[Ca^{2+}]_i$ of iHCM iPSC-CMs more than 5% (Figure 4B). Pyr3, a TRPC3 channel inhibitor,12) was the most potent compound that was effective to both morphological and physiological disease-specific phenotypes of iHCM iPSC-CMs. Notably, Btp2, another TRPC inhibitor was not at all effective to both morphological and physiological disease-specific phenotypes of iHCM iPSC-CMs, suggesting that the tar-
Figure 5. Pyr3 normalizes disease-specific phenotypes of infantile HCM iPSC-CMs with distinct etiology. A: Representative images of iPSC-CMs from iHCM and HCMRAF1, treated with Pyr3 (3 μM) for 5 days and stained with cTnT and Hoechst. Scale bar, 1mm. B: Quantification of the cell area. iPSC-CMs from control (Ctrl), iHCM and HCMRAF1 were treated with Pyr3 for 5 days and the cell area was analyzed by high-content imaging system (n = 5 each). C: iPSC-CMs from control (Ctrl), iHCM and HCMRAF1 were treated with Pyr3 for 5 days and the levels of diastolic [Ca2+]i were quantified (n = 4 each). D: Same number of iPSC-CMs from control (Ctrl), iHCM and HCMRAF1 were treated with Pyr3 for 5 days and the number of remaining iPSC-CMs were counted by high-content imaging system. The cell number relative to vehicle (DMSO)-treated Ctrl iPSC-CMs was presented by bar chart. E: Dose-response curve of isoproterenol (ISO)-induced hypertrophy. iPSC-CMs from iHCM (n = 4 for each concentration) were treated with various concentration of ISO together with Pyr3, and the cell area was analyzed by high-content imaging system. Y-axis represents the fold-change from the iPSC-CMs treated with vehicle instead of ISO and Pyr3. The error bar represents SD. F: EC50 was calculated from the dose-response curve in E. The bar shows the mean and the error bar represents 95% confidence interval. G: Dose-response curve of ISO-induced elevation of diastolic [Ca2+]i, iPSC-CMs from iHCM (n = 3 for each concentration) were treated with various concentration of ISO together with Pyr3 and the levels of diastolic [Ca2+]i were quantified. Y-axis represents the percentage change of diastolic [Ca2+]i from the iPSC-CMs treated with vehicle instead of ISO and Pyr3. H: EC50 was calculated from the dose-response curve in G. The bar shows the mean and the error bar represents 95% confidence interval. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 for comparisons within the iPSC-CMs from the same iPSCs (versus DMSO-treated sample of indicated cell lines), #P < 0.05, ##P < 0.01, ####P < 0.0001 for comparisons between the iPSC-CMs from different iPSCs (versus DMSO-treated control (Ctrl) iPSC-CMs); N.S. indicates not significant in one-way ANOVA followed by Dunnett’s post hoc test. The bar shows the mean, and the error bar represents SD.
get of Pyr3 in normalizing the disease-specific phenotype of iHCM iPSC-CMs is distinct from TRPC.

**Pyr3 normalizes disease-specific phenotypes of infantile HCM iPSC-CMs with distinct etiology:** We finally investigated the reproducibility of the effects of Pyr3 on iHCM and HCM\(^{C3}\) iPSC-CMs. Pyr3 dose-dependently decreased the cell area (Figure 5A, B) in both iHCM and HCM\(^{C3}\) iPSC-CMs. Pyr3 also dose-dependently decreased the diastolic [Ca\(^{2+}\)] in both iHCM and HCM\(^{C3}\) iPSC-CMs (Figure 5C), although the effect on HCM\(^{C3}\) iPSC-CMs did not reach statistical significance due to relatively poor disease-related phenotype in HCM\(^{C3}\) iPSC-CMs. Pyr3 did not affect the cell viability in both iHCM and HCM\(^{C3}\) iPSC-CMs (Figure 5D), suggesting that the effects on cell area and diastolic [Ca\(^{2+}\)], are independent from the toxicity of Pyr3. Pyr3 also decreased the sensitivity of iHCM iPSC-CMs against isoproterenol (Figure 5E-H). Pyr3 also did not affect the beating rate of both iHCM and HCM\(^{C3}\) iPSC-CMs (data not shown). These results collectively suggest that the Pyr3 or its derivative may exhibit therapeutic potential against infantile HCM, irrespective of the etiology, and also suggest that the target molecule of Pyr3 compound may play crucial roles in regulation of both cellular size and calcium homeostasis in cardiomyocytes.

**Discussion**

In the present study, we established the iPSCs from two patients with infantile HCM, one with totally unknown etiology and the other with Noonan syndrome, and characterized the shared disease-specific phenotypes in the patients’ iPSC-CMs. We also performed phenotypic screening using the iPSC-CMs and identified a compound that can normalize both the morphological and physiological disease-specific phenotypes in the iPSC-CMs from both patients.

Establishment of iPSCs from the patients with HCM is already reported from several groups.\(^8\)\(^{-}^{11}\) Disease-specific phenotypes suggesting hypertrophic change of the cardiomyocytes; i.e., enlargement of the cell size and activation of nuclear factor of activated T cells (NFAT) signaling, is observed in the iPSC-CMs from patients with LEOPARD syndrome, one of the variant of Noonan syndrome that exhibits infantile HCM.\(^9\) In addition to those hypertrophic changes, arrhythmia and increased diastolic [Ca\(^{2+}\)] were observed in the iPSC-CMs of HCM patients with MYH7 mutation (Arg663His).\(^9\) Another study also reported that hypertrophic changes, arrhythmia, and increased diastolic [Ca\(^{2+}\)] were observed in the iPSC-CMs of HCM patients with MYH7 mutation (Arg442Gly).\(^9\) Importantly, these disease-specific changes were almost completely normalized by current therapeutics against HCM, i.e., β-blocker and calcium channel inhibitors. In addition, trichostatin A (TSA), an inhibitor of histone deacetylase, also normalized the disease-specific phenotype of iPSC-CMs with MYH7 mutation (Arg442Gly). In the iPSC-CMs of the HCM patients with MYBPC3 mutation, disease-specific phenotype was not obvious at the baseline condition; however, stimulation with endothelin-1 enlarged the cell size and induced abnormal contraction more prominently in iPSC-CMs of HCM patients.\(^11\) Antagonist of endothelin receptor ET\(_{\alpha}\) normalized the endothelin-1-induced disease-specific phenotypes in those iPSC-CMs.

In the present study, we observed hypertrophic change and increased diastolic [Ca\(^{2+}\)]. However, we never observed spontaneous arrhythmia in both iHCM and HCM\(^{C3}\) iPSC-CMs. We also observed some therapeutic effects in β-blocker, calcium channel inhibitor, ET\(_{\alpha}\) antagonist, and TSA on the hypertrophic change of hiPSC-CMs, but these compounds did not show any effect on normalizing the diastolic [Ca\(^{2+}\)]. As the disease-specific phenotypes were similar in HCM iPSC-CMs with same gene mutation (MYH7) and differed from other gene mutation (MYBPC3), the discrepancies in disease-specific phenotypes among the studies, including our study, may reflect the differences in the type of gene mutation that iPSCs are harboring. In addition, maturity of the iPSC-CMs may also affect the arrhythmia phenotype because shallower resting membrane potential increases the vulnerability against arrhythmia. Increasing the repertory of HCM iPSCs with distinct types of gene mutation may clarify which phenotype is “disease-specific” and which is “genotype-specific.” Furthermore, drug screening using HCM iPSC-CMs that harbors different types of gene mutation may enable us to identify a drug candidate that can treat the patients with HCM with any type of (including unknown) etiology.

In the present study, we have identified Pyr3 as a compound that could normalize both morphological and physiological phenotypes of iPSC-CMs from infantile HCM patients, irrespective of the background genotype/etiology. There is a previous report showing that Pyr3 attenuates the cardiac hypertrophy in pressure-overloaded mice model.\(^12\) We, therefore, assume that Pyr3 acts on the general process of cardiac hypertrophy, not the specific process in hypertrophic cardiomyopathy. Pyr3 is a pyrazole derivative that was originally identified as a highly selective inhibitor of TRPC3. However, later studies identified that Pyr3 also inhibits store-operated calcium entry (SOCE)\(^20\), and the information about the selectivity of Pyr 3 in cardiomyocytes, not gene-overexpressing cell lines, is still lacking. We favor the hypothesis that target of Pyr3 in cardiomyocytes is distinct from TRPC or SOCE because Btp2, another inhibitor of both TRPC and SOCE, did not show any effect (rather showed detrimental effect) on both morphological and physiological disease-specific phenotypes of iPSC-CMs from infantile HCM patients. Knockdown of TRPC3 using siRNA or animal experiments using TRPC3 knockout mice may clarify the role of TRPC3 in the pathogenesis of HCM. Alternatively, investigating the novel target of Pyr3 may unveil the novel molecule that is essential in the pathogenesis of HCM. Moreover, testing the therapeutic effect of Pyr3 in other HCM iPSC-CMs, especially those with MYH7 or MYBPC3 gene mutations, and in animal model of HCM may lead to clinical development of Pyr3 or its derivative in the treatment of HCM.
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Disclosures

Conflicts of interest: The authors declare no conflict of interests.

References


Supplemental Files

Supplemental Figures 1, 2
Please see supplemental files: https://doi.org/10.1536/ihj.17-730