Modeling Treatment Response for Lamin A/C Related Dilated Cardiomyopathy in Human Induced Pluripotent Stem Cells

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Background—Precision medicine is an emerging approach to disease treatment and prevention that takes into account individual variability in the environment, lifestyle, and genetic makeup of patients. Patient-specific human induced pluripotent stem cells hold promise to transform precision medicine into real-life clinical practice. Lamin A/C (LMNA)-related cardiomyopathy is the most common inherited cardiomyopathy in which a substantial proportion of mutations in the LMNA gene are of nonsense mutation. PTC124 induces translational read-through over the premature stop codon and restores production of the full-length proteins from the affected genes. In this study we generated human induced pluripotent stem cells-derived cardiomyocytes from patients who harbored different LMNA mutations (nonsense and frameshift) to evaluate the potential therapeutic effects of PTC124 in LMNA-related cardiomyopathy.

Methods and Results—We generated human induced pluripotent stem cells lines from 3 patients who carried distinctive mutations (R225X, Q354X, and T518fs) in the LMNA gene. The cardiomyocytes derived from these human induced pluripotent stem cells lines reproduced the pathophysiological hallmarks of LMNA-related cardiomyopathy. Interestingly, PTC124 treatment increased the production of full-length LMNA proteins in only the R225X mutant, not in other mutations. Functional evaluation experiments on the R225X mutant further demonstrated that PTC124 treatment not only reduced nuclear blebbing and electrical stress-induced apoptosis but also improved the excitation-contraction coupling of the affected cardiomyocytes.

Conclusions—Using cardiomyocytes derived from human induced pluripotent stem cells carrying different LMNA mutations, we demonstrated that the effect of PTC124 is codon selective. A premature stop codon UGA appeared to be most responsive to PTC124 treatment. (J Am Heart Assoc. 2017;6:e005677. DOI: 10.1161/JAHA.117.005677.)

Key Words: dilated cardiomyopathy • lamin A/C cardiomyopathy • nonsense mutation • PTC124 • translational read through
Effects of PTC124 on LMNA-Related Cardiomyopathy

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Cardiac Differentiation

Cardiac differentiation of hiPSCs was induced using a previously reported protocol.²² In brief, undifferentiated hiPSCs were maintained in mTeSR1 medium (STEMCELL Technologies Inc, Vancouver, BC, Canada). Four days before induction, hiPSCs were dissociated into single cells with accutase (Invitrogen, Carlsbad, CA) and then seeded onto a 12-well matrigel-coated plate (Thermo Scientific Inc, Waltham, MA) supplemented with Y27632 (5 μmol/L) (Stemgent, Cambridge, MA). On the first induction day the culture medium was switched to RPMI medium (Life Technologies, Carlsbad, CA) without insulin and supplemented with B27 (Life Technologies) and a GSK-β inhibitor, CHIR99021 (12 μmol/L) (Selleckchem, Houston, TX), and refreshed 24 hours later. On day 4, a Wnt signaling inhibitor, IWP2 (5 μmol/L) (Selleckchem, Houston, TX), was added to the culture medium. Typically, spontaneously beating cardiomyocytes were

Methods

Generation of hiPSC From Patients Carrying Cardiomyopathy-Associated LMNA Mutations

The study protocol for procurement of human tissue for the generation of hiPSCs was approved by the local Institutional Review Board and was registered at the Clinical Trial Center, the University of Hong Kong (IRB-UW08-258). After obtaining written informed consent from all participants, we collected skin biopsies. Skin biopsy samples were mechanically dissociated and plated onto 6-well culture dishes with the culture medium supplemented with 10% fetal bovine serum, as previously described.²¹ Dermal fibroblasts growing out from the skin tissue were expanded and transduced with lentiviruses encoding human OCT4, SOX2, KLF4, and c-MYC. Putative hiPSC clusters were typically observed at 14 to 21 days after lentiviral transduction and were manually dissected and expanded onto new matrigel-coated 6-well plates. The authenticity of the hiPSCs was confirmed with the expression of a panel of pluripotent markers, transgene silencing, OCT4 promoter demethylation, and teratoma formation after inoculation into severe combined immunodeficiency mice (data not shown). All stem cell characterization works have been reported previously.²¹

Clinical Perspective

What Is New?

- This is the first study of the read-through drug PTC124 in patient human induced pluripotent stem cells-derived cardiomycocytes for treatment of nonsense (UGA stop codon–specific) related cardiac laminopathy by recovery of expression of full-length Lamin A/C.

What Are the Clinical Implications?

- This study demonstrates the potential feasibility of human induced pluripotent stem cells-based precision medicine approaches to predict clinical responses of dilated cardiomyopathy patients and to study disease mechanisms.
- Drug testing in patient human induced pluripotent stem cells-derived cardiomyocytes may be a possible approach to select PTC124 responders for entry into clinical trials.

PTC124 on lamin A/C protein production, nuclear senescence, and cell apoptosis. Not only will our results allow us to differentiate a PTC124 responder from nonresponder to enter the clinical trials, but, more importantly, they demonstrate the feasibility of this iPSC-based precision medicine approach in studying genetic diseases.
observed around 9 days after induction. The cells were maintained in the cardiomyocyte maintenance medium (RPMI medium with B27 supplement) and used in subsequent analyses.

**LMNA-Related Cardiomyopathy Model**

The hiPSC-based model of LMNA-related cardiomyopathy was induced using electrical stimulation according to our previously published protocol. Specifically, hiPSC-derived cardiomyocytes were seeded on 13-mm glass coverslips (Nunc A/S, Rockilde, Denmark) and mounted on a 6-well plate filled with corresponding medium. Electrical stimulation was then delivered to the cultured cells for 4 hours with carbon electrodes using an 8-channel C-Pace cell culture stimulator (IonOptix Co, Milton, MA) at 6.6 V/cm, 1 Hz, 2 milliseconds with alternating polarity. The total number of cells was counted before and after electrical stimulation.23-25

**PTC124 Treatment**

A stock solution of PTC124 was prepared by dissolving the lyophilized PTC124 (Selleckchem, Houston, TX) in DMSO to a concentration of 10 mmol/L. The stock solution was diluted in cell culture medium and preheated to 37°C to ensure complete dissolution before being applied to the cells. To test the potential therapeutic effects of PTC124, hiPSC-derived cardiomyocytes were pretreated with PTC124 (50 μmol/L) for 7 days before electrical stressing.

**Western Blot Analysis**

Western blot analysis was performed following the protocol described previously.26 In brief, cells of interest were washed with Dulbecco phosphate-buffered saline and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA) containing 0.2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin with additional 100 mmol/L NaF and 2 mmol/L Na3VO4. The supernatant was collected after spinning for 20 minutes at 12,000g. The amount of protein was quantified using a Bio-Rad protein assay kit (Hercules, CA). For each sample, 50 μg of total protein was resolved on a 4% to 12% Bis-Tris plus gel (Gibco, Gaithersburg, MD) and transferred onto nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBS (pH 7.4) with 0.5% Tween-20 at 4°C, the transferred protein was probed by primary mouse monoclonal antibodies specific to lamin A/C (clone 4C-11; 1:2000; Cell Signaling Technologies, Danvers, MA). As an endogenous control, the levels of β-actin were evaluated using a monoclonal antibody specific to β-actin. The location of the immunoreactive protein complex was detected with HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA; 1:2000) and visualized with standardized enhanced chemiluminescence systems.

**Assessment of Nuclear Blebbing Events**

At 30 to 40 days after induction of differentiation, hiPSC-derived cardiomyocytes were dissociated by collagenase treatment and subsequently seeded on gelatin-coated coverslips for immunostaining analysis and functional evaluation. For immunostaining, cells were fixed and permeabilized in 2% paraformaldehyde for 20 minutes at 4°C and then washed with wash buffer (Dulbecco phosphate-buffered saline with 0.1% Triton X-100; Sigma-Aldrich, St. Louis, MO). The nuclear lamina of hiPSC-derived cardiomyocytes was visualized by staining with antibodies specific to lamin A/C (clone 4C-11; 1:200; Cell Signaling Technologies, Danvers, MA) and cardiact-specific markers such as goat anti–tropomin-T (1:400; Abcam, Cambridge, UK). Positively probed cells were visualized by addition of rabbit anti–goat IgG H+L Alexa 594 and rabbit anti–mouse IgG H+L Alexa 488 (Molecular Probes, Eugene, OR) for 30 minutes. Images were acquired using a fluorescence confocal Carl Zeiss LSM 700 microscope (Zeiss GmbH, Gottingen, Germany).

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay for Apoptosis**

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using an in situ Cell Death Detection Kit, Fluorescein (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer’s protocol. Cells were grown on glass coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Cells were then washed in 0.1% sodium citrate for 2 minutes on ice. Cells were then washed in 0.1% sodium citrate for 2 minutes on ice. Cells were then washed in 0.1% sodium citrate for 2 minutes on ice. Cells were then washed in 0.1% sodium citrate for 2 minutes on ice. Cells were then washed in 0.1% sodium citrate for 2 minutes on ice. Cells were incubated in TUNEL reaction mixture at 37°C for 60 minutes in a humified dark chamber. The TUNEL-labeled samples were subjected to costaining with tropomin-T antibodies for confirmation of cardiac identity. Coverslips were mounted onto glycerol-based mountant. Images were acquired using a Carl Zeiss fluorescence microscope with AxioVision 6.0 software (Zeiss GmbH, Gottingen, Germany). The number of apoptotic cells on electrical stimulation was further quantified using the APO-BrdU TUNEL Assay Kit (Molecular Probes, Inc, Eugene, OR). The dissociated cells were fixed in 1% (w/v) paraformaldehyde on ice for 15 minutes and then washed twice with Dulbecco phosphate-buffered saline at 300g for 5 minutes. The fixed cells were stored in ice-cold 70% (v/v) ethanol at −20°C before the nicked end labeling reaction. The cells were washed with wash buffer before incubation in TUNEL labeling solution for an hour at 37°C (10 μL reaction buffer, 0.75 μL TdT enzyme, 8 μL of BrdU, and 31.25 μL dH2O). After further washing with rinse buffer, cells were
stained with the Alexa Fluor® 488 dye-labeled anti-BrdU antibodies for a half-hour. The propidium iodide/RNase A staining buffer was finally added to the cells before flow cytometry analysis. The positively labeled cells (green signal), a population with DNA fragmentation, were counted as percentage of apoptotic cells.

**Taqman Assay for Quantification of LMNA Expression**

Total RNA was extracted from fibroblasts using TRIZOL reagent following the procedure provided by the manufacturer (Life Technologies, Carlsbad, CA). For each sample, 1 µg of total RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription kit (Qiagen, Venlo, The Netherlands). Taqman gene expression assay was carried out using a FAM-labeled probe derived from the Universal Probe Library (lamin A/C (LMNA): probe #66), troponin-T type 2 (TNNT2): probe #63 and α-tubulin (TUB4a): probe #75 (Roche Applied Sciences) in combination with FastStart Universal Probe Master (Rox) as the detection reagent (Roche Applied Sciences, Indianapolis, IN). The samples were analyzed as duplicates on the StepOnePlus real-time polymerase chain reaction system (Life Technologies). TUB4A served as a housekeeping gene for normalization, and TNNT2 was used for normalization of cardiac purity within the whole population.

**Excitation-Contraction Coupling Analysis by Simultaneous Calcium Imaging and Video Edge Detection**

To study the calcium-handling properties, hiPSC-derived cardiomyocytes were loaded with Fura-2 AM (Life Technologies, Carlsbad, CA), a calcium indicator, at a working concentration of 1 µM for 20 minutes. Unincorporated dye was removed, and the cells were subsequently incubated in Tyrode solution. For calcium transient measurements, field-stimulated electrical pacing was induced by the Myopacer EP Field Stimulator (IonOptix, Westwood, MA) at 40 V cm⁻¹, 5-millisecond pulse duration at frequencies of 0.5 Hz, 1 Hz, 1.5 Hz, and 2 Hz. The calcium fluorescence and video-edging (contractile force) signal were simultaneously recorded by MyoCam-S (IonOptix) using IonWizard 6.4 version 2 software (IonOptix). The acquisition rate of calcium imaging was 100 points/s. The Fura-2 required dual-wavelength excitation at 340 nm and 380 nm, and the emission signals were recorded at 505 nm. The calcium level was presented as a ratio of 340/380 nm (F340/F380) and calibrated with amount of free calcium (nmol/L). The calcium transients of every cardiomyocyte were recorded with background subtraction.

**Statistical Analysis**

Continuous variables are expressed as mean±SEM. Statistical comparisons between 2 groups were performed using Student t test or nonparametric Mann-Whitney test (for n<10). For analysis involving more than 2 groups, 1-way ANOVA analysis was used. For posttest, Tukey analysis was used to compare all pairs of columns. A P value <0.05 was considered statistically significant.

**Results**

**Patient Characteristics, Generation of LMNA-Mutation-Harboring hiPSCs, and Cardiac Differentiation**

Two patients with nonsense LMNA mutations and 1 patient with a frame-shift mutation were recruited to the study. The first patient was a 49-year old Chinese man who initially presented with atrial fibrillation and complete atrioventricular block and required permanent cardiac pacemaker implantation. Two years later, he developed sustained ventricular tachyarrhythmia and heart failure with an impaired left ventricular ejection fraction of 35%. An automatic implantable cardioverter defibrillator was therefore implanted (Table). His family history was remarkable for complete atrioventricular block and sudden cardiac death. Sequencing of the LMNA gene revealed a heterozygous single-base exchange (672G→T) in exon 4, resulting in an R225X nonsense mutation.

<table>
<thead>
<tr>
<th>LMNA Mutations</th>
<th>Sex</th>
<th>Complete Heart Block</th>
<th>AF</th>
<th>VT/VF</th>
<th>Dilated Cardiomyopathy</th>
<th>AICD</th>
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<tr>
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<td>+ (49)</td>
<td>+ (50)</td>
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<td>+ (52)</td>
</tr>
<tr>
<td>Q354X</td>
<td>M</td>
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<td>+ (50)</td>
<td>+ (56)</td>
<td>+ (50)</td>
<td>+ (50)</td>
</tr>
<tr>
<td>T518fs</td>
<td>M</td>
<td>+ (43)</td>
<td>+ (43)</td>
<td>+ (47)</td>
<td>+ (47)</td>
<td>+ (47)</td>
</tr>
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</table>

AF indicates atrial fibrillation; AICD, automatic implantable cardioverter defibrillator; VT/VF, ventricular tachycardia/ventricular fibrillation.

*+* represents presence of disease phenotype, while the ages of disease onset are shown in the brackets.
(truncation in the coil 1B domain of both lamin A and C proteins), previously known to be associated with familial dilated cardiomyopathy18,27,28 (Figure 1A and 1B). The second patient was a 50-year old Chinese man who also had atrial fibrillation, complete atrioventricular block, ventricular tachyarrhythmia, and heart failure. Of his 4 siblings, 3 had similar cardiac conditions, and all had had an automatic implantable cardioverter defibrillator implanted. A heterozygous single-base exchange (1062C→T) in exon 6 of the LMNA gene resulting in a Q354X nonsense mutation was found to be cosegregated with the cardiac phenotype in this family (Figure 1). The last patient was a 43-year-old Chinese man with atrial fibrillation, complete atrioventricular block, and ventricular tachyarrhythmia. A heterozygous single-base deletion (1554delC) at exon 9 of the LMNA gene was detected. According to our prediction of a second open reading frame with 1 “C” deletion at codon position 1554 in the LMNA mRNA transcript (NM_170707), the premature stop codon with sequence of “TGA” will be generated at an earlier position at the 547th amino acid, shortening the LMNA protein that is supposed to be transcribed (reduced from 665 to 547 amino acids) (Figure S1). This patient’s paternal uncle

![Figure 1](http://jaha.ahajournals.org/)

**Figure 1.** Schematic diagram illustrating the LMNA mutations involved in this study. A, Genetic disorders caused by nonsense and frame-shift mutations in LMNA. R225X yielded a premature stop codon, TGA, due to a C-to-T substitution; Q354X also yielded a premature stop codon, TAG, due to a C-to-T substitution; and the frameshift mutation T518fs resulted from a C deletion. B, Splice variant of LMNA gene yielding lamin A/C protein: the mutation yielded 3 different lengths of truncated lamin A/C protein. Key: NLS indicates nuclear localization signal.
had had atrial fibrillation and complete atrioventricular block (Figure 1A and 1B). Dermal fibroblasts were obtained from these patients during automatic implantable cardioverter defibrillator implantation, and patient-specific hiPSCs harboring the specific mutations were generated accordingly. The patient-specific LMNA mutations in these hiPSCs had been confirmed by genomic DNA sequencing analysis. For simplicity in description, the hiPSC lines containing the 672G→T, 1062C→T, and 1554delC mutations in 1 of the LMNA alleles will be denoted as R225X, Q354X, and T518fs mutants, respectively, in this article.

The commercially available hiPSC line (line IMR90, Wicell) and KS1 hiPSC line generated by our lab by lentiviral reprogramming that do not contain any LMNA mutations was used as a wild-type control in this study 21 (Figure 1).

Effects of PTC124 on Expression of Lamin A/C Proteins in Dermal Fibroblasts and hiPSC-Derived Cardiomyocytes

First, we tested the effects of PTC124 on the level of lamin A/C proteins in dermal fibroblasts obtained from the 3 patients

Figure 2. Effects of PTC124 on the expression of lamin A/C proteins in dermal fibroblasts and hiPSC-derived cardiomyocytes (hiPSC-CMC) derived from wild-type (LMNA WT1/WT1 and LMNA WT2/WT2) and LMNA mutants (LMNA R225X/WT, LMNA Q354X/WT, and LMNA T518fs/WT). A, Representative immunoblot and (B) quantitative data of protein-normalized lamin A/C protein expression. The band intensities of lamin A/C and β-actin (Actb) were measured from the high-resolution (300×300 dots per inch) images using Image J. At least 3 to 6 independent samples were prepared for Western blot analysis. The significant difference of quantitative data was tested by 2-way ANOVA with post hoc Turkey multiple comparison test (**P<0.01 as indicated by arrows; #P<0.05, Control vs PTC). Original images of immunoblots in individual experiments are shown in Figure S2. hiPSC indicates human induced pluripotent stem cells.
Figure 3. Nuclear blebbing in the hiPSC-derived cardiomyocytes. The 2 wild-type lines (LMNA\(^{WT1/WT1}\) and LMNA\(^{WT2/WT2}\)), R225X (LMNA\(^{R225X/WT}\)), Q354X (LMNA\(^{Q354X/WT}\)), and T518fs (LMNA\(^{T518fs/WT}\)), mutant hiPSC lines were differentiated into cardiomyocytes and treated with PTC124. The occurrence of cardiac nuclear blebbing was revealed with coimmunostaining of cardiac troponin T (red) and lamin A/C (green). A, A single representative hiPSC CMC, and (B through D) representative images of lower magnification were used to quantify the portion of nuclear blebbing in a single field. At least 3 countings were performed. E, Quantitative data of nuclear blebbing count. The significant difference of quantitative data was tested by 2-way ANOVA with post hoc Turkey multiple comparison test (**P<0.0001 as indicated by arrows; ####P<0.0001 control vs treatment group). cTnT indicates cardiac troponin-T; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; hiPSC, human induced pluripotent stem cells; LMNA, lamin.
with LMNA-related cardiomyopathy (Figure 2). Dermal fibroblasts were treated with PTC124 (50 μmol/L), and the lamin A/C protein levels were evaluated by Western blotting analysis. In the absence of PTC124, the translation yielded only intact lamin A/C products: a 74-kDa band indicating lamin A protein and a 63-kDa band from lamin C protein, in
dermal fibroblasts from all 3 patients. No truncated lamin A/C protein was detected in any of the mutant lines (data not shown) (Figure 2). Compared with their wild-type counterpart, the levels of lamin A/C proteins were markedly reduced in dermal fibroblasts from all mutant lines. Treatment with PTC124 increased the level of both lamin A and C isoforms in dermal fibroblasts carrying the R225X mutation in the LMNA gene but not in the wild-type or dermal fibroblasts from the other 2 patients harboring Q354X or T518fs mutations. Importantly, as no prolonged lamin A/C protein products were detected, PTC124 treatment was not likely causing normal termination codon read-through.

Similar results were observed in the hiPSC-derived cardiomyocytes treated with PTC124. In brief, the level of lamin A/C proteins in all 3 mutant lines was substantially reduced compared with their wild-type counterpart in the absence of PTC124 (Figure 2). Similar to the findings observed in dermal fibroblasts, PTC124 treatment increased the level of both lamin A and C proteins in cardiomyocytes derived from the R225X mutant hiPSC line but not in those of the other 2 LMNA mutations (ie, Q354X and T518fs mutants). Interestingly, mild accumulation of prelamin A was observed in the cardiomyocytes derived from Q354X and T518fs mutants (Figure 2), regardless of the presence of PTC124 treatment.

**Effects of PTC124 on Electrical Stimulation–Induced Nuclear Senescence and Apoptosis in hiPSC-Derived Cardiomyocytes**

As previously reported by our team and others, the nuclei of cardiomyocytes from LMNA-related cardiomyopathy often exhibit dysmorphic features such as micronucleation (blebbing), one of the hallmarks of nuclear senescence. These changes together with apoptosis are often exaggerated in cardiomyocytes that are electrically active or under constant electrical stress. As shown in Figure 3, coimmunostaining of lamin A/C (green) and cardiac troponin-T (red) revealed that the cardiomyocytes derived from R225X or T518fs mutant lines exhibited nuclear blebbing even in the absence of external stress. After continuous electrical stressing, the nuclear blebbing events became more frequent in all the hiPSC-derived cardiomyocytes carrying LMNA mutations. Interestingly, the application of PTC124 alleviated nuclear senescent morphological abnormalities in the cardiomyocytes derived from the R225X mutant but not in those derived from the Q354X or T518fs mutant lines (Figure 3A through 3D). To evaluate the effects of PTC124 on apoptosis, we first performed TUNEL analysis. As shown in Figure 4A through 4D, in concordance with the nuclear blebbing assay, the electrical stress-induced apoptosis in cardiomyocytes with R225X mutation was markedly reduced in the presence of PTC124 (Figure 4B). Nonetheless, no significant changes in apoptosis were detected on application of PTC124 in the other 2 mutants (Figure 4C through 4D). In order to provide more quantitative assessment of the protective effects of PTC124 against electrical stress-induced apoptosis, we further quantified the proportion of apoptotic cells using the APO-BrdU TUNEL assay (Figure 4F through 4G and Table S1). Compared with the wild-type control, all 3 mutants showed a significantly higher proportion of apoptotic cardiomyocytes. On electrical stressing, the proportion of apoptotic cells increased markedly by 40.58% (n=3-7, P<0.05) only in hiPSC-derived cardiomyocytes with the R225X mutation (Figure 4G). More importantly, application of PTC124 significantly reduced the electrical stimulation-induced apoptotic population by 41.89% in cardiomyocytes derived from R225X mutant (n=3-5; P<0.05). Neither electrical stress nor the application of PTC124 caused any statistically significant changes to the proportion of apoptotic cells in any of the other groups.

**Nonsense LMNA mRNA Degradation**

In addition to defective translation as a result of the nonsense mutations, the suppressed production of lamin
Figure 4. Evaluation of TUNEL-positive apoptotic cell in electrically stressed and PTC124-treated cardiomyocytes derived from wild-type \( \text{LMNA}^{\text{WT}1/\text{WT}1} \) and \( \text{LMNA}^{\text{WT}2/\text{WT}2} \) and \( \text{LMNA} \) mutants (\( \text{LMNA}^{\text{R225X/WT}} \), \( \text{LMNA}^{\text{Q354X/WT}} \), and \( \text{LMNA}^{\text{T518fs/WT}} \)) by (A through E) immunostaining; (F through G) and also by Apo-BrdU TUNEL-FACS analysis to quantify apoptotic cells (Significant difference was analyzed by 2-way ANOVA with Tukey multiple comparison post-hoc test *\( P<0.05; n=3 \) to 5 (as indicted by arrows) and (control vs electrically stressed) \( \#P<0.05; n=3 \) to 7). FACS indicates fluorescence-activated cell sorting; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
A/C proteins could be due to a nonsense-mediated mRNA decay mechanism.\textsuperscript{31} To evaluate such a possibility, we precisely quantified the \textit{LMNA} mRNA level in cardiomyocytes derived from the wild-type and mutant lines using \textit{Taqman} real-time polymerase chain reaction assay. As shown in Figure 5, the relative cardiac \textit{LMNA} expression in all 3
mutants was significantly lower than that in the wild-type control. This suggests that nonsense mRNA degradation may contribute to the reduced lamin A/C protein level.

Interestingly, PTC124 could not restore the mRNA production in any mutant line, suggesting that its activity was limited to a translational level.

Figure 4. Continued
Effects of PTC124 on Excitation-Contraction Coupling in Cardiomyocytes Derived From the R225X Mutant hiPSC

Because the PTC124-mediated reduction in apoptosis was observed only in the cardiomyocytes derived from the R225X mutant and not in other lines, we further evaluated the effects of PTC124 on excitation-contraction coupling, calcium homeostasis, and contractile abnormalities in this particular line. Contractilities and calcium transients were recorded simultaneously under field electrical pacing at frequencies ranging from 0.5 Hz to 2 Hz. As shown in Figure 6A, hiPSC-derived cardiomyocytes (both wild-type and mutants) were responsive to electrical pacing and showed comparable diastolic and peak calcium levels (Figure 6B and 6D). Diastolic calcium levels (indicated by the baseline levels of the calcium transient tracings) of both lines increased as the pacing frequency increased (Figure 6B). In general, the maximum calcium released (indicated by the peak amplitude of the calcium transient tracings) in cardiomyocytes derived from the R225X mutant was less than that released in the wild-type counterparts (Figure 6C). The systolic cell length also reduced remarkably in the mutant cardiomyocytes treated with PTC124, indicating a more efficient coupling between intracellular calcium surge and cellular contraction (Figure 6E). Regardless of the application of PTC124, there was no significant change in the upstroke velocity (ie, the rate of calcium release) of calcium transients or rate of cell shortening between wild-type and mutant hiPSC-derived cardiomyocytes (Figure 6F and 6G). Nevertheless, at high pacing speed (2 Hz), PTC124 significantly improved the rate of calcium reuptake in the mutant cardiomyocytes (as indicated by a more negative value of $V_{\text{max}}$ decay) ($P<0.05$, n=3) (Figure 6H). Furthermore, PTC124 treatment appeared to improve the rate of cellular relengthening in the mutant cardiomyocytes (Figure 6I).

Discussion

Precision medicine, according to the National Institutes of Health in the United States, is an emerging approach for disease treatment and prevention that takes into account
Excitation-contraction (EC) coupling was improved by PTC124 in the cardiomyocytes derived from the R255X mutant. A, Representative tracing of calcium transient and cell-shortening in LMPA<sup>WT/WT</sup> and LMPA<sup>R225X/WT</sup> cells treated with PTC124. Rate dependence of calcium transients and cell contractility pacing frequency range of 0.5, 1, and 1.5 to 2 Hz are shown in terms of (B) diastolic calcium, (C) peak calcium amplitude, (D) fractional shortening (%) (*<i>P</i>< 0.05; n=5 to 7); WT: PTC vs R225X: P<0.0001; R225X: 0.5-Hz control vs R225X: 0.5-Hz or 1-Hz PTC: <i>P</i> < 0.001; R225X: 0.5 Hz PTC vs R225X: 1.5 or 2 Hz PTC: <i>P</i> < 0.001); (E and F) maximal upstroke velocity (<i>V</i><sub>max</sub> upstroke) of calcium transient and rate of cell shortening (ie, contraction); (G and H) maximal decay velocity (<i>V</i><sub>max</sub> decay) and rate of cell relengthening (ie, relaxation). Improved calcium decay kinetics and relaxation performance are shown (G and H). Significant difference was analyzed by comparing pacing rate of 0.5 Hz with the other rates of the same treatment group in 1 cell line, or otherwise indicated by arrows using 2-way ANOVA with Turkey test as post hoc (*<i>P</i>< 0.05).
individual variability in environment, lifestyle, and genetic makeup for individual patients. The assumption is that differences in an individual patient’s genetic makeup as well as environmental factors contribute to his or her differential clinical outcome, particularly in responsiveness to treatments. In other words, patients with apparently similar clinical conditions may be treated differently because of these individual differences. Over the past 3 decades, there has been tremendous progress in genetics research that has identified numerous genes responsible for dilated cardiomyopathies and provided novel insight into their pathogenic mechanisms. Nonetheless, translation of these research findings to clinical practice remains incomplete. In fact, even with the availability of an individual patient’s genetic information that underlies dilated cardiomyopathies, a disease-specific intervention designed to tackle the root causes of the condition is rarely available. As a result, despite different genetic etiologies and pathogeneses, dilated cardiomyopathies are often treated similarly simply based on clinical presentations.

Mutations in LMNA genes are the most common cause of familial dilated cardiomyopathy, particularly among those with concomitant atrioventricular conduction block. In our study, despite different mutations in the 3 patients with LMNA-related cardiomyopathy, their clinical presentations were remarkably similar with early onset atrioventricular block and atrial fibrillation around the fourth decade of life, followed by ventricular tachyarrhythmia and heart failure in the fifth to sixth decade of life. The condition was inherited in an autosomal dominant pattern in all 3 families. In accordance with their genotypes, both dermal fibroblasts and cardiomyocytes derived from R225X, Q354X, and T518fs mutants displayed reduced lamin A/C protein production. As demonstrated in our previous hiPSC-based model of LMNA-related cardiomyopathy, cardiomyocytes derived from these 3 mutants exhibited nuclear senescence and apoptosis that were aggravated in the presence of electrical stress. Nonetheless, despite their very similar clinical presentations and cellular phenotypes, there was a differential effect of PTC124 on lamin A/C protein production in their primary dermal fibroblasts (R225X > Q354X and T518fs). Similar to dermal fibroblasts, PTC124 restored lamin A/C protein production only in cardiomyocytes derived from the R225X mutants but not in their Q354X or T518fs counterparts. More importantly, the increase in lamin A/C production was associated with alleviated nuclear senescence and reduced apoptosis. Furthermore, PTC124 treatment improved the excitation-contraction coupling and contractile functions of the hiPSC-derived cardiomyocytes carrying the R225X mutation.

The lack of responsiveness to PTC124 of cardiomyocytes derived from the T518fs mutant, which served as a negative control, is expected. On the contrary, for the cardiomyocytes derived from the Q354X mutant, the lack of efficiency could be explained by the difference in nucleotide sequence of the mutant premature stop codons. Nonsense mutations are...
point mutations that result in the occurrence of 1 of 3 stop codons, UAG, UAA, or UGA, in the messenger RNA coding region, leading to a truncated protein product due to premature termination of mRNA translation and/or promotion of mRNA destabilization by nonsense-mediated mRNA decay. Although PTC124 promotes read-through of all 3 nonsense codons, its potency differs according to the nucleotide sequence of the premature stop codons, with the highest read-through at UGA, followed by UAG, and then UAA. For the 2 nonsense mutations, the R225X mutation in the LMNA gene is a result of a premature stop codon (UGA) that is the most PTC124-responsive nucleotide sequence, whereas in the Q354X mutant, the sequence of the stop codon is UAG, which seems to be less PTC124 responsive. Because the efficacy of the translational read-through of premature stop codons and production of full-length proteins by interfering with ribosomal proofreading depend on the availability of nonsense transcripts to the ribosomal translational machinery, it is interesting that in our study, application of PTC124 increased mRNA expression of the 2 nonsense transcripts: R225X and Q354X. Nonetheless, it is beyond the scope of the present study to determine whether PTC124 can affect the efficiency of the nonsense mutation mRNA decay mechanisms.

For nonsense mutation, it seems to be stating the obvious that translational read-through of premature stop codons with PTC124 is expected to produce full-length proteins and reverse the pathogenic process. Nonetheless, in a recent study using hiPSC-derived cardiomyocytes from 2 patients who harbored nonsense mutations in the sodium channel gene SCN5A, R1638X and W156X, although the premature stop codon sequences in both mutations were the most optimum sequence for PTC124, UGA, PTC124 and gentamicin, another premature stop codon read-through agent, failed to restore sodium current at Na%. Although the sodium channel protein level was not quantified to document the restored protein translation, other downstream mechanisms such as a trafficking defect might have been responsible. This reinforces the need for precision medicine. hiPSC technology and in vitro drug testing strategies provide unparalleled opportunities to realize the promise of precision medicine. This strategy may be exploited to select the patients with maximum drug responsiveness for standard clinical trials. Then, the expected drug effects may be much larger, and the required sample size would then be much smaller, making standard randomized clinical trials possible.

Conclusions

Taken collectively, PTC124 promoted translation read-through of premature stop codons in patient-specific hiPSC-derived cardiomyocytes that harbored the mutation R225X in the LMNA gene and alleviated nuclear senescence, apoptosis, and altered excitation-contraction coupling. The same effects were not evident in patient-specific hiPSC-derived cardiomyocytes that harbored another nonsense mutation or a frame-shift mutation. Based on our data, hiPSC technology represents a general approach to predict the clinical response of patients with an apparently similar clinical condition and to realize the potential of precision medicine.

Acknowledgments

We are thankful for the technical support provided by Dr Au.

Disclosures

None.

References

Effects of PTC124 on LMNA-Related Cardiomyopathy  Lee et al


SUPPLEMENTAL MATERIAL
Table S1. Raw data of apoptotic assay of the effects of PTC124 on electrically-stressed hiPSC-CMs by Apo-BrdU-TUNEL FACS analysis

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<td>826</td>
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**Figure S1.**

**A**

Translation of *LMNA* wild type transcript (NM_170707)

```
1  agc gag acc cag tcc cag cag cgg cpc qoc acq cgc cpc gcc ggg cag cag
27  Met Glu Thr Pro Ser Gin Arg Arg Ala Thr Arg Ser Cys Ala Gin
45
16  Ala Ser Ser Thr Pro Leu Ser Ser Pro Thr Arg Ile Arg Leu Gin
30
91  gcg aag gwg gcg cag gwg ctc aat gat cpg tgg gcg tgc ttc
135  Gin Lys Gin Arg Leu Gin Gin Leu Arg Arg Arg Leu AlaVal Tyr
153
157  atc gcg cgt qgc tgc tgc gaa agq agq acq cgg cgg cag cgc
180  Ile Asp Gin Gin Val Leu Thr Gin Leu Gin Gin Gin Leu Gin Gin
205
311  ctc cpp acc gag cag ggc gac ggc gac ggg gcg tcc aag ggc cgg
335  Leu Arg Ala Arg Ser Arg Ser Arg Ser Gly Gin Gin Gin Gin Gin
360
526  gag arg cag ggc tgc ggg aac agc tgg gaa gaa gtg gcc atg
540  Glu Asp Leu Arg Arg Ser Arg Ser Asn Ser Val Glu Glu Glu Val Gin
565
106  leu ser lye val arg glu glu phe lye lye lye ala arg am
130
211  acc aag acq ggc gcq cgq tcc cag cag tcg cgc atc cgc atc gac
235  Thr Leu Gin Gin Lys Gin Gin Gin Gin Gin Gin Gin Gin Gin
259
436  thr ser gly arg val ala val glu glu val asp glu glu gly lys
460
761  cgc aag ctg gtg cgc tca gtg act gtg gtt gag gac gac gag gat
785  Lys Glu Lys Gin Gin Lys Gin Gin Gin Gin Gin Gin Gin Glu Glu
810
1126  tac ggc agq ctg cag gag agq atg gag atg antic cgc cgc
1150  Tyr Arg Asp Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg
1174 317  ggc atc aag gcc gcc
341
```

**Yellow:** Start codon  
**Blue:** Frameshift mutation site due to deletion  
**Red:** Stop codon
### B Translation of LMNA T518 frameshift mutation transcript

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<th>Codon</th>
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<td>Met</td>
<td>Start codon</td>
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<td>7</td>
<td>End</td>
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**Premature stop codon**

Yellow: Start codon
Blue: Frameshift mutation site due to deletion
Red: Stop codon
Figure S2. A.
B

Skin fibroblast
6/12/2016
SF5: R225X;
Siu-1: WT
C

Skin fibroblast
SF5: R225X;
Siu-1: WT

Lamin A/C  N-18
(N-terminal)

Lamin A/C 636
(C-terminal)
Skin fibroblast
19/6/12
Sf30 = Q354X

Lamin A/C N-18 (N-terminal)

Q354X Ptc 0 Ptc 50
R225X Ptc 0 Ptc 50

ACTB
Skin fibroblast
SF36: R225X
SF30: Q354X
Skin fibroblast
27/03/2017
WT-1: Siu1
WT-2: IMR
Lamin A/C  N-18 (N-terminal)

Skin fibroblast
27/03/2017
WT-2: IMR
R225X
Ptc 0 Ptc 50

T518fs
Ptc 0 Ptc 50

Lamin A/C  N-18
(N-terminal)

ACTB

19/8 hiPSC-CM
Sf 36 = Q354X
Sf 39 = T518fs
6/9 hiPSC-CM
Wt
Sf36 = R225X
Sf30 = Q354X
Sf39 = T518fs

Lamin A/C N-18 (N-terminal)

ACTB
Lamin A/C N-18
(N-terminal)

hiPSC-CM
31/03/2017
WT-2: IMR
Supplemental Figure Legends:

**Figure S1. Translational product of LMNA T518fs mutated transcript.** (A) Wild type LMNA mRNA transcript (NM_170707); (B) Prediction of second open reading frame (ORF) with one “c” deletion at the 1554th codon position in the LMNA mRNA transcript, the premature stop codon with sequence of “TGA” will be generated at an earlier position at the 547th amino acid, shortening of the Lmna protein that is supposed to be transcribed (reduced from the 665th to 547th amino acids). Moreover, even after PTC124 read-through, the amino acid sequence after frameshift mutation (the 519th amino acid) would be totally deviated from the native lamin A/C ones.

**Figure S2. Effects of PTC124 on the expression of lamin A/C proteins in dermal fibroblasts (A-H) and hiPSC-derived cardiomyocytes (hiPSC-CMC) (I-M) derived from wild-type (LMNAWT1/WT1 & LMNAWT2/WT2) and LMNA mutants (LMNAR225X/WT, LMNAQ354X/WT and LMNAT518fs/WT).** Original images of immunoblots with at least one wild type sample loaded on the same gel with patient samples. Lamin A/C (clone N18) were probed with antibody that recognized N-terminal, while beta-actin (ACTB) was used as the internal control for normalization of protein loading. Three to six independent samples were prepared for western blot analysis.
Modeling Treatment Response for Lamin A/C Related Dilated Cardiomyopathy in Human Induced Pluripotent Stem Cells
Yee-Ki Lee, Yee-Man Lau, Zhu-Jun Cai, Wing-Hon Lai, Lai-Yung Wong, Hung-Fat Tse, Kwong-Man Ng and Chung-Wah Siu

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