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ORIGINAL RESEARCH ARTICLE

Unfolded Protein Response as a Compensatory Mechanism and Potential Therapeutic Target in PLN R14del Cardiomyopathy

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BACKGROUND: Phospholamban (PLN) is a critical regulator of calcium cycling and contractility in the heart. The loss of arginine at position 14 in PLN (R14del) is associated with dilated cardiomyopathy with a high prevalence of ventricular arrhythmias. How the R14 deletion causes dilated cardiomyopathy is poorly understood, and there are no disease-specific therapies.

METHODS: We used single-cell RNA sequencing to uncover PLN R14del disease mechanisms in human induced pluripotent stem cells (hiPSC-CMs). We used both 2-dimensional and 3-dimensional functional contractility assays to evaluate the impact of modulating disease-relevant pathways in PLN R14del hiPSC-CMs.

RESULTS: Modeling of the PLN R14del cardiomyopathy with isogenic pairs of hiPSC-CMs recapitulated the contractile deficit associated with the disease in vitro. Single-cell RNA sequencing revealed the induction of the unfolded protein response (UPR) pathway in PLN R14del compared with isogenic control hiPSC-CMs. The activation of UPR was also evident in the hearts from PLN R14del patients. Silencing of each of the 3 main UPR signaling branches (IRE1, ATF6, or PERK) by siRNA exacerbated the contractile dysfunction of PLN R14del hiPSC-CMs. We explored the therapeutic potential of activating the UPR with a small molecule activator, BiP (binding immunoglobulin protein) inducer X. PLN R14del hiPSC-CMs treated with BiP protein inducer X showed a dose-dependent amelioration of the contractility deficit in both 2-dimensional cultures and 3-dimensional engineered heart tissues without affecting calcium homeostasis.

CONCLUSIONS: Together, these findings suggest that the UPR exerts a protective effect in the setting of PLN R14del cardiomyopathy and that modulation of the UPR might be exploited therapeutically.

Key Words: cardiomyopathy, dilated ■ induced pluripotent stem cells ■ models, biological ■ phospholamban ■ sequence analysis, RNA ■ unfolded protein response

Phospholamban (PLN) encodes a critical regulatory protein of Ca^{2+} cycling and is a primary mediator of the β-adrenergic effects, resulting in enhanced cardiac output.[1] The levels of PLN and its degree of phosphorylation profoundly influence the activation state of the sarcoplasmic reticulum calcium ATPase (SERCA2a). In the dephosphorylated state, PLN interacts with SERCA2a and shifts its Ca^{2+} activation toward lower apparent Ca^{2+} affinity. On protein kinase A–mediated phosphorylation, the inhibitory interaction between PLN and SERCA2a is abolished, and the apparent Ca^{2+} affinity is raised. Thus, PLN is the rate-determining factor for

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Clinical Perspective

What Is New?

- Using human induced pluripotent stem cells and CRISPR-Cas9 gene editing technologies, we created an in vitro model of dilated cardiomyopathy associated with the phospholamban (PLN) R14del mutation.
- Single-cell RNA sequencing revealed the activation of the unfolded protein response (UPR) pathway in PLN R14del human induced pluripotent stem cell–derived cardiomyocytes.
- UPR activation was also evident by significant upregulation of marker gene in the hearts of patients with PLN R14del.
- Pharmacological and molecular modulation of the UPR suggests a compensatory role in PLN R14del dilated cardiomyopathy.
- Augmentation of UPR by the small molecule BiP (binding immunoglobulin protein) inducer X ameliorated contractile dysfunction.

What Are the Clinical Implications?

- The findings suggest a mechanistic link between proteostasis and PLN R14del-induced pathophysiology that could be exploited to develop therapeutic strategies for PLN R14del cardiomyopathy.
- Targeting of the UPR might be a viable therapeutic strategy for the treatment of PLN R14del dilated cardiomyopathy.
- The study highlights human induced pluripotent stem cell–derived cardiomyocyte modeling combined with small molecule testing as a paradigm for studying genotype-phenotype associations in heart disease with the potential for the development of mechanism-based therapeutic strategies.

Nonstandard Abbreviations and Acronyms

BiP	binding immunoglobulin protein
BiX	binding immunoglobulin protein inducer X
CASQ2	calsequestrin 2
DCM	dilated cardiomyopathy
EHTs	engineered heart tissues
ER	endoplasmic reticulum
HD	healthy donor
hiPSC	human induced pluripotent stem cell
hiPSC-CM	human induced pluripotent stem cell–derived cardiomyocyte
NCX1	sodium-calcium exchanger
R14del	deletion of the arginine 14 codon in the PLN gene
RYR2	ryanodine receptor 2
scRNA-seq	single-cell RNA sequencing
SERCA2a	sarcoplasmic reticulum calcium ATPase
UPR	unfolded protein response
WT	wild-type

Ca^{2+} reuptake by SERCA2a and a key regulator of contractility in the heart.

Dilated cardiomyopathy (DCM) is the leading cause of heart failure, and familial DCM is responsible for up to a third of the reported cases.[2] Various pathogenic genetic variants have been linked to DCM, including mutations in the coding region of the *PLN* gene. DCM caused by the deletion of the arginine 14 codon in the *PLN* gene (R14del) is associated with prevalent ventricular arrhythmias, heart failure, and sudden cardiac death.[3,4] The limited mechanistic understanding of how the R14del contributes to the overall clinical presentation translates to the lack of disease-specific therapeutic strategies.

In this study, we investigated the molecular underpinnings of PLN R14del-induced cardiomyopathy by leveraging the power of human induced pluripotent stem cells (hiPSCs), CRISPR/Cas9 genome editing, and single-cell RNA sequencing (scRNA-seq) technologies. PLN R14del hiPSC-derived cardiomyocytes (hiPSC-CMs) faithfully recapitulated the contractile dysfunction observed in PLN R14del-induced cardiomyopathy. At the molecular level, we observed an elevated endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in the PLN R14del hiPSC-CMs compared with isogenic controls. Molecular and pharmacological modulation of the UPR pathway revealed a protective role of the UPR activation in PLN R14del hiPSC-CMs. Our findings suggest a mechanistic link between proteostasis and PLN R14del-induced pathophysiology that could be exploited to develop therapeutic strategies for PLN R14del cardiomyopathy.

METHODS

Patients and healthy control subjects were enrolled in the study by informed consent approved by the Stanford Institutional Review Board and Stem Cell Research Oversight Committee. The histopathological part of the study met the criteria of the code of proper use of human tissue that is used in the Netherlands. The collection of human cardiac tissue was approved by the scientific advisory board of the biobank of the University Medical Center Utrecht, Utrecht, the Netherlands (protocol 12/387).

A detailed description of the Methods is available in the Data Supplement. The data, analytical methods, and study materials are available to other researchers for purposes of reproducing the results or replicating the procedures on request. The hiPSC lines have been deposited to the Stanford's Cardiovascular Institute iPSC Biobank and are available on request.

Statistical Analysis

Data were processed and analyzed with Microsoft Excel and GraphPad Prism version 8.1.1 (GraphPad). For statistical analysis of 2 data sets, the Student *t* test was used. One-way ANOVA was used with the Tukey multiple-comparisons test to evaluate statistical differences among groups. Factorial designs were analyzed with 2-way ANOVA for repeated measures in case of serial measurements, with adjustment by the Tukey post hoc

test in case of multiple comparisons. Data plots are displayed as mean±SEM unless specified otherwise, and a value of $P<0.05$ was set to determine statistical significance.

Data Availability

Because of the sensitive nature of the data collected for this study, requests to access the data set from qualified researchers may be sent to the corresponding author.

RESULTS

Modeling DCM Contractility Defect With hiPSC-CMs

To model PLN R14del DCM, we recruited 2 unrelated families carrying the mutation (Figure I in the Data Supplement) to generate hiPSCs from carriers. Isogenic control lines in which the genetic background is identical are important for unequivocal assignment of phenotype to the underlying gene variant. Therefore, we corrected the R14del mutation in both patient hiPSC lines through CRISPR/Cas9–mediated genome editing and similarly introduced the R14del mutation into an hiPSC line derived from an individual with no history of heart disease (healthy donor [HD]; Figure 1A and 1B). We generated 3 pairs of isogenic models that differ only in the PLN R14del mutation (Figure 1C, Figure II, and Tables I-IV in the Data Supplement). Impaired contractility is a pathological hallmark of DCM; therefore, we assessed the contractile function of the hiPSC-CMs. Consistent with previous studies,[5] hiPSC-CMs carrying the PLN R14del mutation (patient and HD R14del introduced) showed decreased contractility in 3-dimensional engineered heart tissues (EHTs; Figure 1D and 1E) and 2-dimensional monolayer cultures (Figure 1F and 1G). Therefore, the isogenic paired hiPSCs lines recapitulated the contractile deficit, providing a quantifiable model for determining the molecular mechanisms that underlie the development of DCM.

scRNA-seq Analysis Reveals UPR Activation

Single-cell transcriptomic analysis makes it possible to deconvolute the complex transcriptional responses that occur naturally across populations of cells into clusters of similarly responding cells. We used a high-throughput droplet-based scRNA-seq method (10X Genomics) to examine the transcriptional effects caused by introducing the PLN R14del mutation into an HD hiPSC line. Unbiased *t*-distributed stochastic neighbor embedding clustering parsed 9244 single-cell transcriptomes from PLN wild-type (WT) hiPSC-CMs (5279 cells) and PLN R14del hiPSC-CMs (3965 cells) into 10 distinct subpopulations (Figure 2A). The heterogeneity of the cardiomyocyte cultures was similar to previous scRNA-seq studies.[6] Despite this heterogeneity, *t*-distributed stochastic neighbor embedding analysis revealed close clustering of the PLN mutant line with its isogenic counterpart, indicating that the transcriptomes and cell type composition are preserved across isogenic populations (Figure 2B). To assign cellular identity, subpopulations were classified on the basis of known marker genes. Most of the cell clusters appeared to be cardiomyocytes based on their specific patterns of gene expression (clusters 1–4 and 6–7 expressed marker genes *TNNT2*, *MYL2*, and *MYH7*). Two clusters, clusters 5 and 8, were identified as fibroblasts (expressing marker genes *COL3A1*, *COL1A1*, and *FN1*), and 1 cluster with very few cells, cluster 9, was identified as smooth muscle cells (with marker genes *ACTA2* and *TAGLN*; Figure 2C and 2D).

Next, we explored the transcriptional changes associated with the R14del mutation. An advantage of single-cell over bulk RNA sequencing is that it affords greater statistical power to resolve differential expression because it focuses within cell-identity clusters rather than across the broader heterogeneous population in which cell type–specific information remains obscure. On the basis of the *t*-distributed stochastic neighbor embedding clustering and canonical marker expression, we restricted the analysis to the cardiomyocyte subclusters 1 to 4 and 6 to 7. Seventy-seven genes (Wilcoxon rank-sum test) were differentially expressed in PLN R14del hiPSC-CMs compared with the isogenic control hiPSC-CMs (Figure 2E). Gene enrichment analysis revealed genes associated with pathways related to ER stress and UPR signaling pathway (Figure 2F). Further bioinformatics analysis showed that several hallmark genes related to UPR pathway were activated in PLN R14del hiPSC-CMs, including gene members of the protein disulfide isomerase (*PDIA3* and *PDIA4*), heat shock protein 90 (*HSP90*A1 and *HSP90B1*), and *HSPA5* (also known as BiP [binding immunoglobulin protein]) compared with isogenic PLN WT controls. Comparison of the gene expression profiles of the cell cluster identified as fibroblasts showed no significant differences in the expression of the UPR hallmark genes between the PLN R14del and PLN WT cells (Figure 2G). We further corroborated the activation of UPR markers in the hiPSC-CMs at the protein level using Western blot analysis (Figure 2H). Finally, we monitored the activity of the UPR pathway in living hiPSC-CMs using an XBP1-splicing reporter construct[7] (Figure 2I) and observed a significant increase in UPR activity in patient PLN R14del cells. Because adrenergic drive activates UPR response in cardiomyocytes,[8,9] we also measured the UPR reporter activity on isoproterenol stimulation. Under adrenergic stress conditions (72 hours of treatment with 1 μmol/L), we noticed an increase in UPR signaling in PLN WT cells but an exaggerated (4-fold higher) response in hiPSC-CMs containing the mutation. Taken together, these data suggest that the PLN R14del mutation activates the UPR transcriptional program and sensitizes R14del hiPSC-CMs to adrenergic stress.

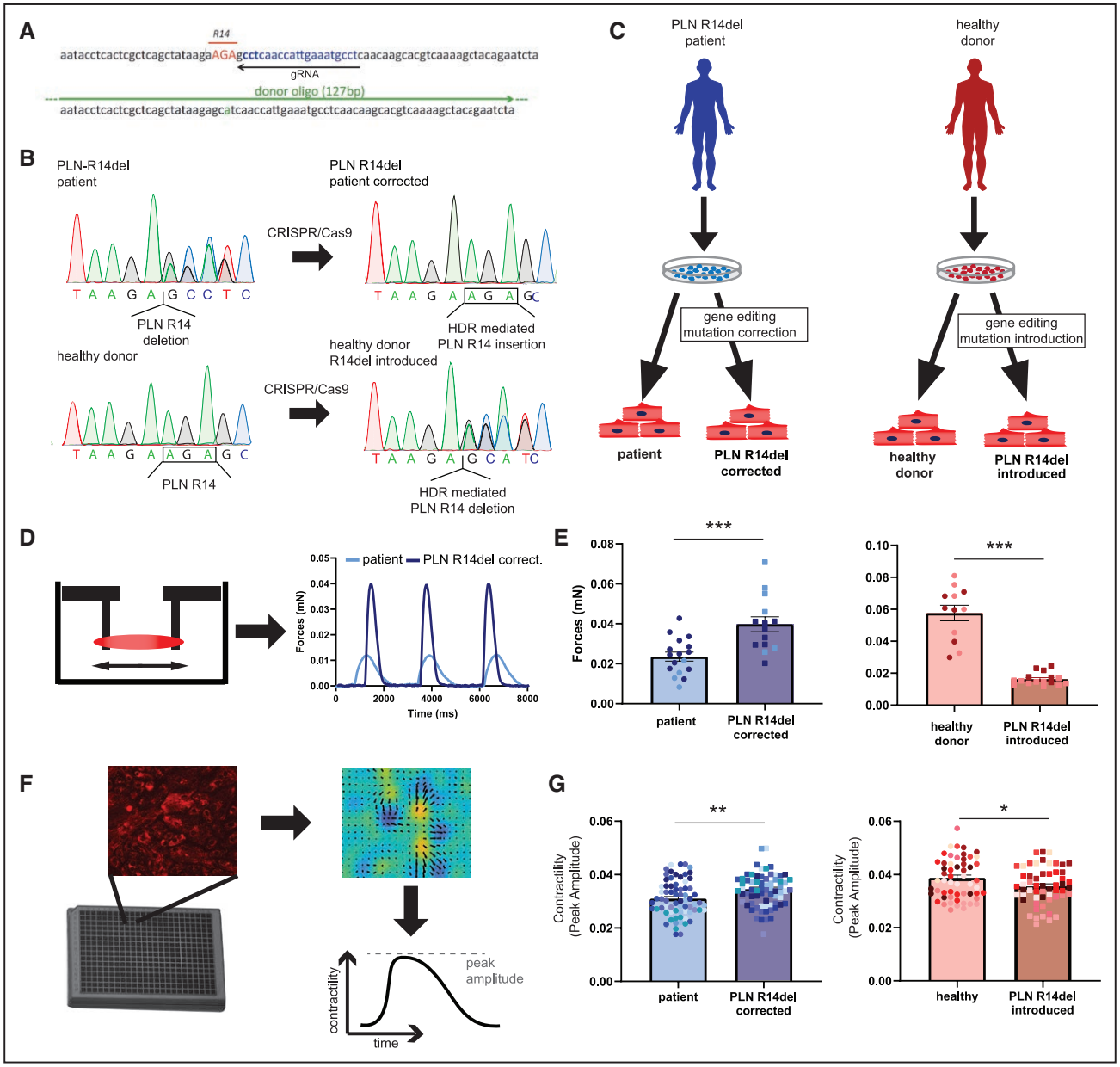

Figure 1. Phospholamban (PLN) loss of arginine at position 14 (R14del) human induced pluripotent stem cell (hiPSC) disease modeling and functional assessment of contractility.

A, Schematic overview of the strategy to precisely modify the *PLN* sequence using CRISPR/Cas9 and single-stranded donor oligonucleotides complementary to the guide RNA (gRNA). **B**, Sanger sequencing analysis showing the correction and introduction of the R14del variant sequence in hiPSCs generated from dilated cardiomyopathy and healthy individual hiPSCs, respectively. **C**, Schematic representation of hiPSC lines used in the study. **D** and **E**, Assessment of force generation of hiPSC–derived cardiomyocytes (CMs) carrying the PLN R14del mutation and their corresponding isogenic controls in 3-dimensional engineered heart tissues (EHTs; 2 batches, n=5–12 EHTs each). **F** and **G**, Two-dimensional monolayer contractility measurements of hiPSC-CMs carrying the PLN R14del mutation and their corresponding isogenic controls (12 batches, n=5–10 wells each). Colors represent experimental batches. Data are presented as mean±SEM. HDR indicates homology-directed repair. **P*<0.05; ***P*<0.005; ****P*<0.0005.

Histopathologic Characterization of the UPR in PLN-R14del Patient Myocardium

The accumulation of protein aggregates is often associated with dysfunctional UPR responses, for instance, in neurodegenerative diseases.[10] Protein aggregation has been noted as a histopathologic characteristic of end-stage PLN R14del DCM hearts but not in hearts representative of other forms of DCM.[11–13] Therefore, we examined human myocardial samples of PLN R14del hearts for evidence of UPR involvement. We also analyzed myocardial tissue from desmosomal arrhythmogenic right ventricular cardiomyopathy and ischemic cardiomyopathy hearts (Figure III and Table V in the Data Supplement). Consistent with previous studies,[13] PLN immunolabeling revealed perinuclear aggregations in

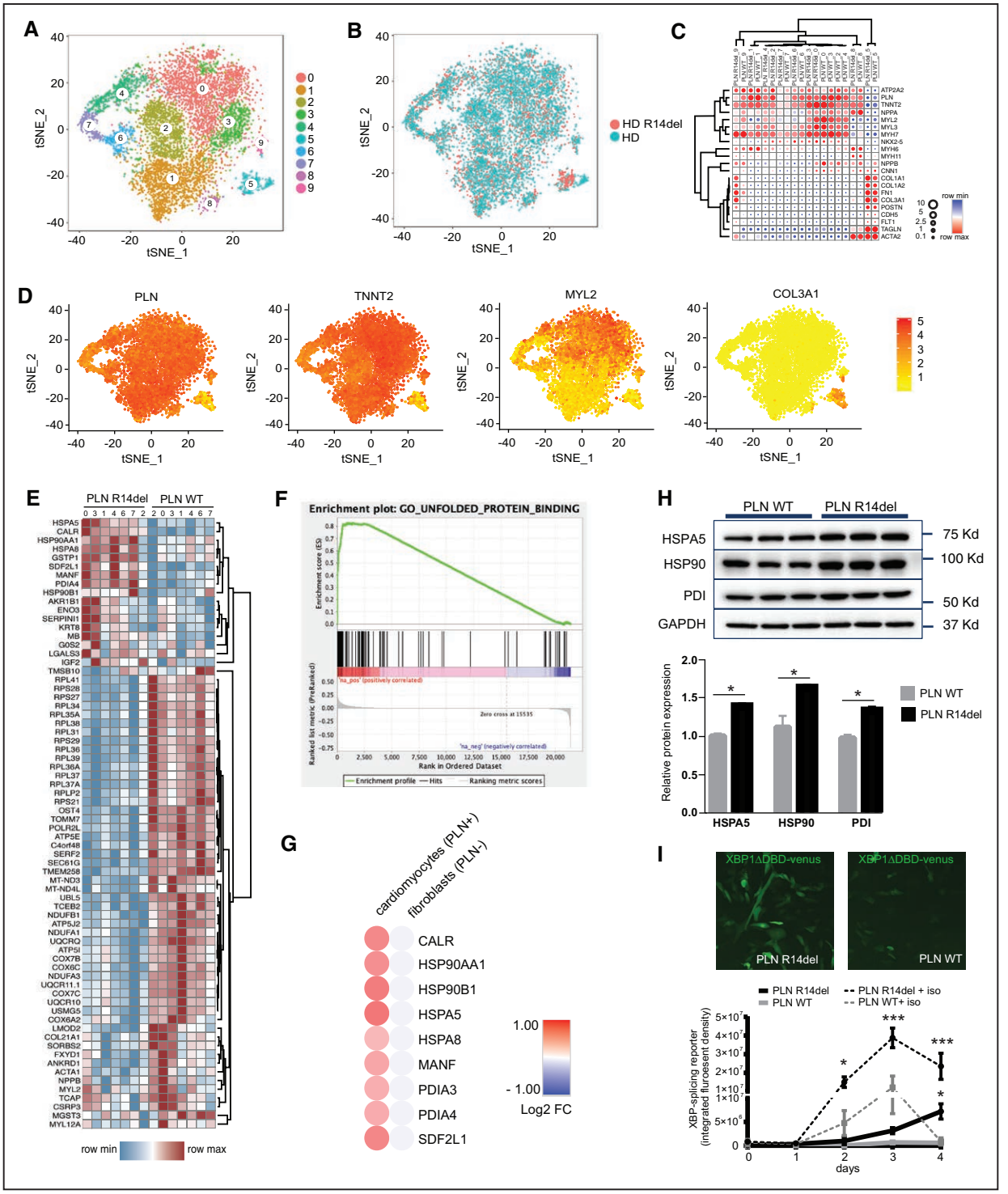

Figure 2. Single-cell RNA sequencing of isogenic human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) carrying the phospholamban (PLN) loss of arginine at position 14 (R14del) mutation.

A and **B**, Unbiased identification of cell clusters using *t*-distributed stochastic neighbor embedding (tSNE)–based clustering of single-cell transcriptomes showing a 2-dimensional visualization with distinctly isolated cell subpopulations (n=5279 cells, healthy donor [phospholamban wild-type (PLN WT)]; n=3965 cells, healthy donor PLN R14del introduced [PLN R14del]). **C** and **D**, Subpopulations were classified according to canonical marker gene expression. **E**, Heat map display of 77 differentially expressed genes in the cardiomyocyte subpopulations. **F**, Gene set enrichment analysis pathway enrichment analysis. **G**, Comparison of unfolded protein response (UPR) hallmark differential gene expression between cardiomyocyte and noncardiomyocyte subpopulations in a healthy donor (HD) and R14del introduced hiPSC-CMs. **H**, Western blot expression analysis of UPR proteins from paired isogenic hiPSC-CM lines (n=3 batches). **I**, Assessment of the UPR activity in living hiPSC-CMs (PLN R14del and PLN WT) transduced with AAV-F-*XBP1*ΔDBD (3 batches, n=4–6 wells each). Statistical significance represented as differences between PLN WT and PLN R14del in either control or isoproterenol (iso) conditions. Data are presented as mean±SEM. **P*<0.05; ****P*<0.0005.

PLN R14del tissues (7±2%), but these aggregations were absent in arrhythmogenic right ventricular cardiomyopathy, ischemic cardiomyopathy, and control hearts (Figure 3A–3D and 3M). BiP is an essential regulator of the UPR that monitors ER stress, and its expression is markedly increased in the presence of unfolded proteins in the ER.[14,15] Accordingly, we observed the highest BiP levels in PLN R14del (23±5%), but we also detected comparable BiP expression in desmosomal arrhythmogenic right ventricular cardiomyopathy (16±5%) and ischemic cardiomyopathy (17±2%) relative to control hearts (2±1%; Figure 3E–3H and 3M), consistent with UPR activation in failing hearts.[16–18] Protein disulfide isomerase, a protein folding facilitator, is another chaperone that is induced by protein misfolding.[19] A striking protein disulfide isomerase presence was detected in the myocardium of patients with PLN R14del (46±29%) relative to subjects with desmosomal arrhythmogenic right ventricular cardiomyopathy (7±6%), those with ischemic cardiomyopathy (6±4%), and control subjects (3±3%; Figure 3I–3M). In addition, RNA sequencing showed that UPR genes are upregulated in the myocardium of R14del patient cardiac tissue relative to that of healthy individuals (Figure 3N). Taken together, the histological manifestations of PLN R14del share hallmarks of neurodegenerative disease wherein the accumulation of protein aggregates is associated with abnormal levels of ER stress response.[20,21]

UPR Activity Protects Contractile Function in hiPSC-CMs Harboring PLN-R14del

In principle, UPR could have a pathological or beneficial effect on the PLN R14del cardiomyocytes. To establish the role of the UPR, we carried out a loss-of-function experiment in which we attenuated the main transducers of the UPR (IRE1, ATF6, and PERK; Figure 4A). These interconnected signaling branches of the UPR provide the cells with an adaptive response to ER stress to restore proteostasis.[22] Under baseline conditions, targeting each of the 3 branches by siRNA did not significantly affect the contractility in either PLN WT (HD and patient R14del corrected) or PLN R14del (patient and HD R14del introduced) hiPSC-CMs, although a trend toward decreased function (≈10%) was observed with siRNA against IRE1 and PERK in PLN R14del (Figure 4B and 4C) hiPSC-CMs. Given that long-term adrenergic stimulation dramatically

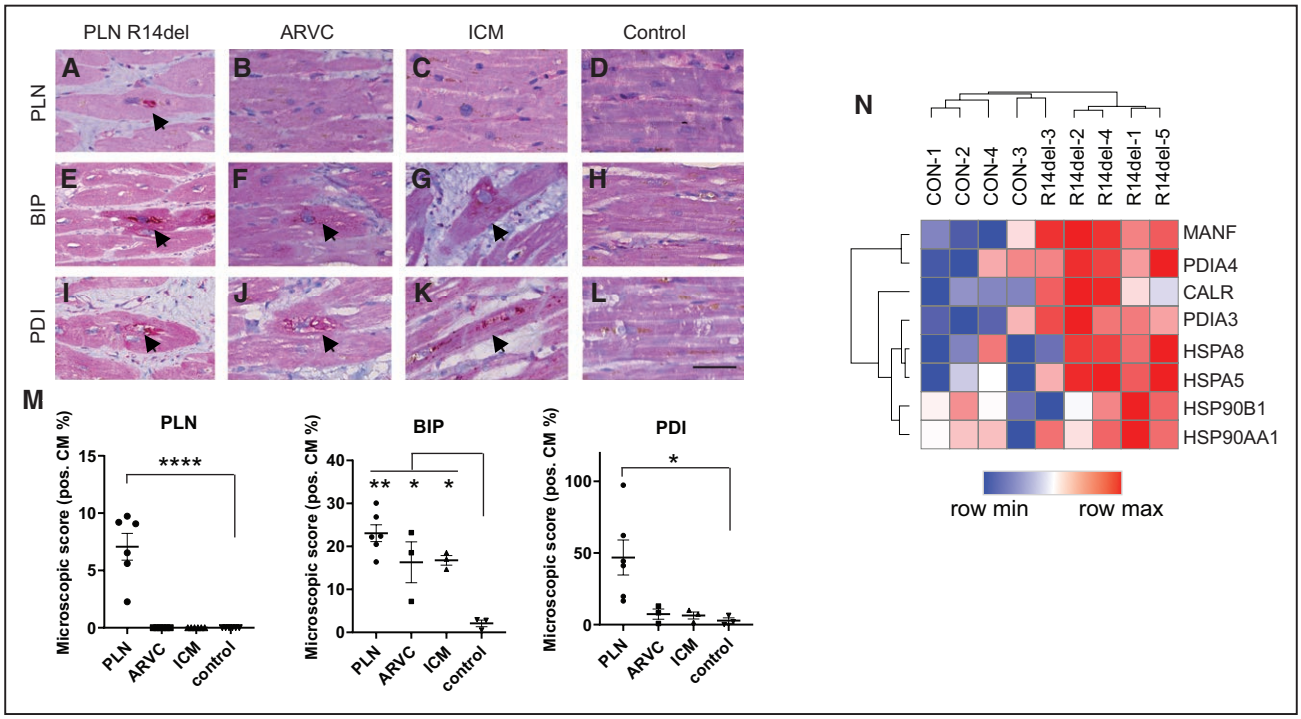

Figure 3. Determination of the unfolded protein response (UPR) status in phospholamban (PLN) loss of arginine at position 14 (R14del) disease and other forms of cardiomyopathies.

Histological analysis of human myocardium from patients with PLN R14del, desmosomal arrhythmogenic cardiomyopathy (ARVC), and ischemic cardiomyopathy (ICM) vs control (CON; healthy) hearts. **A** through **D**, Abnormal accumulation of PLN in perinuclear aggregates (arrows) in severely affected cardiomyocytes (CMs) in PLN R14del and absent in ARVC, ICM, and control. **E** through **H**, Diffuse moderate immunolabeling (arrows) for BiP (binding immunoglobulin protein) in PLN 14del and present to a lesser extent in ARVC and ICM. **I** through **L**, High immunolabeling for dotted cytoplasmic protein disulfide isomerase (PDI; arrows) in PLN 14del and low PDI presence in ARVC and ICM (scale bar=25 µm). **M**, Quantification of immunostaining in PLN R14del (n=6 patients), ARVC (n=3 patients), ICM (n=3 patients), and control (n=3 patients). **N**, RNA sequence analysis of UPR gene expression from healthy and PLN R14del myocardium from patient samples. Data are presented as mean±SEM. **P*<0.05; ***P*<0.005; *****P*<0.00005.

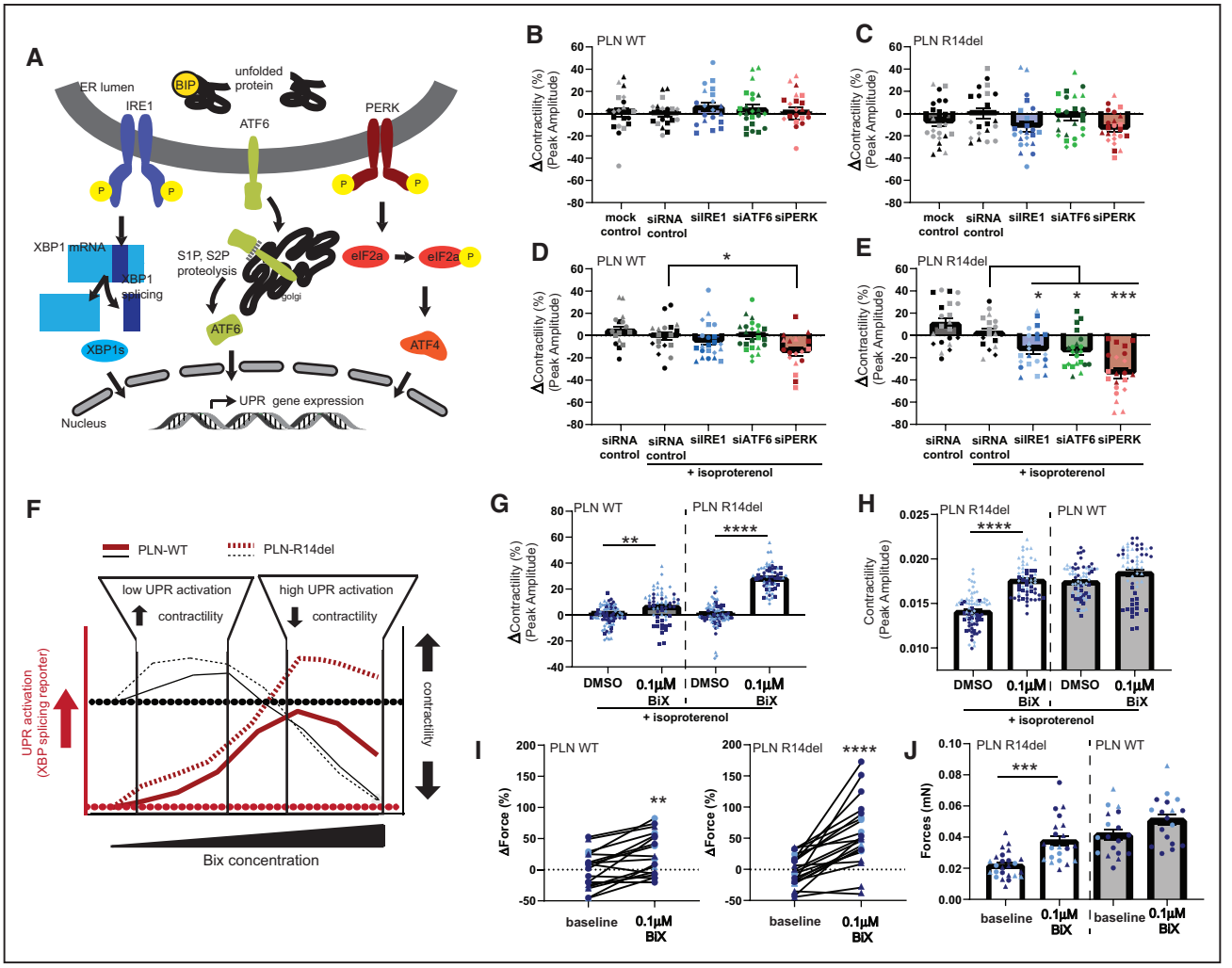

Figure 4. Defining the role of the unfolded protein response (UPR) pathway in human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) with loss of arginine at position 14 (R14del).

A, Schematics of UPR signaling including stress sensors IRE1, ATF6, and PERK at the endoplasmic reticulum (ER) membrane and the 3 corresponding downstream transcriptional effectors. **B** through **E**, Functional analysis of UPR pathway perturbation in isogenic hiPSC-CMs. Cells were transfected with the indicated siRNAs, and 2-dimensional (2D) monolayer contractility was measured after 4 days either under baseline conditions (**B** and **C**) or with 0.1 μmol/L isoproterenol (**D** and **E**). Contractility represented as percent change from control siRNA (6 batches, n = 2–3 wells each). ●,■, ▲, and ◆ represent experimental batches; dark symbols represent hiPSC-CMs from healthy donor background; light symbols represent hiPSC-CMs from the patient background. All data are presented as mean±SEM. **P*<0.05; ****P*<0.0005 vs siRNA controls (**D** and **E**). **F** through **H**, Assessment of contractility in hiPSC-CMs after pharmacological activation of the UPR with the small molecule BiP (binding immunoglobulin protein) inducer X (BiX). Overview of BiX dose-response kinetics in hiPSC-CMs showing effects on UPR activation and contractility (**F**; see also Figure IV in the Data Supplement). Assessment of 2D contractility measurements after 72 hours of BiX (or dimethyl sulfoxide [DMSO] vehicle control) treatment with 0.1 μmol/L isoproterenol in isogenic hiPSC-CMs (**G** and **H**; 4 batches, n=12–16 wells each). Contractility is represented as percent change from the DMSO control per batch (**G**) or actual peak contraction amplitude (**H**). ●,■, ▲, and ◆ represent experimental batches; dark symbols represent hiPSC-CMs from healthy donor background; light symbols represent hiPSC-CMs from the patient background. All data are presented as mean±SEM. ***P*<0.005; *****P*<0.00005 vs DMSO vehicle controls (**G** and **H**). **I** and **J**, Force generation of 3-dimensional engineered heart tissues (EHTs) derived from isogenic hiPSC-CMs at baseline and after treatment with BiX for 72 hours. Force generation is represented as percent change from baseline (**I**) or as total generated force (**J**; 4 batches, n=2–8 EHTs each). WT indicates wild-type. ***P*<0.005; ****P*<0.0005; *****P*<0.00005 by paired Student *t* test.

increased UPR fluxes in hiPSC-CMs harboring PLN R14del (Figure 2I), we next evaluated the impact of the loss of function in the presence of isoproterenol. Under adrenergic stress, the contractility of PLN WT hiPSC-CMs remained unchanged after the siRNA-mediated knockdown of IRE1 and ATF6, but we observed significantly decreased contractile function after PERK knockdown (14.3±3.7%; Figure 4D). In contrast, we observed a significant contractility deficit in PLN R14del hiPSC-CMs upon knockdown of each of the 3 UPR arms compared with control siRNA treated (siRNA against IRE1, −15.5±4.2%; siRNA against ATF6, −16.7±4.1%; siRNA against PERK, −36.6±5%), indicating that the mutant hiPSC-CMs were sensitized to a loss of UPR signaling in a genotype-specific manner (Figure 4E). Taken together, these data suggest

that activation of the UPR in PLN R14del hiPSC-CMs preserves cell function and therefore plays a protective role in alleviating ER stress and potentially blunts disease pathogenesis.

Pharmacological Targeting the UPR in PLN-R14del Ameliorates Contractility

Next, we evaluated whether pharmacologically stimulating the UPR pathway in PLN R14del hiPSC-CMs beyond basal levels would have a positive effect on cardiomyocyte function. BiP protein inducer X (BiX) is a small molecule proteostasis regulator that increases expression of the BiP protein to consequently induce UPR and protect neurons from ER stress.[23,24] In both PLN R14del (patient and HD R14del introduced) and PLN WT (HD and patient R14del corrected) hiPSC-CMs, treatment with BiX induced a dose-dependent increase in a reporter for XBP1-splicing (indicative of activated UPR). The maximal effect was higher in PLN R14del than in corresponding isogenic control hiPSC-CMs (E_{max}=33.1±7% versus 56.8±7% XBP-1 splice-positive cells in normal versus R14del hiPSC-CMs, respectively; Figure IVb and IVc in the Data Supplement). BiX (0.1 μmol/L) significantly enhanced contractility in PLN R14del and PLN WT hiPSC-CMs as detected in 2-dimensional cell sheets (Figure 4G and 4H). In the EHTs, this translated to an increase in force from about 20 μN in untreated EHTs to 40 μN in treated EHTs, corresponding to a level statistically indistinguishable from isogenic control EHTs (Figure 4I and 4J). Remarkably, the increase in peak contractility was more pronounced in the PLN R14del hiPSC-CMs, and BiX treatment (0.1 μmol/L) led to a nearly complete restoration of the contractile deficit without increasing the expression levels of myofilament proteins (Figure V and Table VI in the Data Supplement). At high concentrations, BiX decreased contractility in both the PLN R14del and isogenic controls (E_{max}=−25% at 10 μmol/L for both R14del and isogenic controls; Figure IVa in the Data Supplement). We considered whether the beneficial effect of BiX is mediated through the modulation of the calcium handling properties of PLN R14del hiPSC-CMs. Compared with a dimethyl sulfoxide control, there was no significant difference in the intracellular calcium handling kinetics in PLN R14del hiPSC-CMs treated with BiX (Figure VI in the Data Supplement). In agreement, the expression levels of PLN and other key calcium handling proteins, including SERCA2a, NCX1 (sodium-calcium exchanger), RYR2 (ryanodine receptor 2), and CASQ2 (calsequestrin 2), were similar between BiX and dimethyl sulfoxide–treated PLN R14del hiPSC-CMs (Figure VII and Table VI in the Data Supplement). Collectively, these data suggest that stimulating the UPR pathway in PLN R14del hiPSC-CMs restored contractility to levels of isogenic controls without affecting calcium homeostasis.

DISCUSSION

Human iPSCs provide an opportunity for modeling DCM in vitro to understand the molecular consequences of pathogenic mutation such as PLN R14del. However, the inherited heterogeneity caused by a mixture of cell types in hiPSC-CM differentiation cultures and the genetic background of the donor present confounding factors for defining disease-specific phenotypes. To overcome these challenges, we generated multiple isogenic hiPSC lines carrying the R14del mutation and performed scRNA-seq on these genotype-defined hiPSC lines. This experimental approach can be widely used to define the specific contributions of pathogenic mutation in DCM and cardiomyopathies more broadly.

Using this approach, we revealed that the PLN R14del mutation activates the UPR, an integrative intracellular signaling pathway that plays a critical role in the maintenance of proteostasis in the ER.[25] Proteostasis, the balance among protein synthesis, folding, refolding, and degradation, is essential for the long-term preservation of cell and tissue function. With age, the ability of many cells and organs to maintain proteostasis is gradually compromised.[26] Accumulating evidence suggests that UPR is activated in response to a loss of proteostasis in the ER and the corresponding accumulation of protein aggregates, which characterizes age-related diseases and protein folding disorders such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis.[10] Reminiscent of these diseases, aggregation of PLN protein is a hallmark of PLN R14del DCM,[12] suggesting that the disruption of proteostasis plays a role in disease presentation. It is tempting to speculate that the altered stability and folding kinetics of the PLN R14del mutant directly activate the UPR in mutant hiPSC-CMs and human heart tissues. It will be of interest to explore this hypothesis further in the future.

Our isogenic models allowed us to perform high-throughput genetic and pharmacological assays both to understand the underlying pathological mechanisms and to identify therapeutic targets to prevent or treat such diseases. We found that activation of UPR in PLN R14del hiPSC-CMs is protective because molecular inhibition of each of the 3 UPR sensors, IRE1, PERK, and ATF6, exacerbated the contractile dysfunction. Conversely, pharmacological enhancement of UPR by the small molecule agonist BiX ameliorated the contractility defect in PLN R14del hiPSC-CMs. BiX activates the chaperone BiP, thereby increasing the activity of the UPR pathway. These observations are consistent with previous studies showing that activation of UPR suppressed disease onset and progression in cellular and animal models of neurodegenerative diseases[27–31] and prevented damage in the heart caused by ischemia/reperfusion.[32–34] These findings suggest that enhancing the expression

or activity of individual proteostasis network components could be therapeutically beneficial in PLN R14del DCM.

Here, we showed that the small molecule BiX improved contractile performance without affecting Ca^{2+} transients in PLN R14del hiPSC-CMs. BiX preferentially activates ER stress response elements upstream of the BiP gene, a molecular chaperone, thereby increasing the activity of the UPR pathway.[22] BiX showed a detrimental effect at high doses in hiPSC-CMs, suggesting a narrow therapeutic window between the effective doses and those at which it causes adverse toxic effects. The underlying mechanism for the biphasic response is unclear. It is possible that BiX is a ubiquitous ER stressor, limiting its application as a therapeutic strategy because it may activate several pathways of the UPR, including the ER stress–induced apoptotic pathways. Although the inotropic mechanisms of BiX are unprecedented, our findings suggest that selective modulation of UPR components could improve cardiac function in PLN R14del DCM and potentially avoid the deleterious effects that can occur with drugs that target calcium signaling or other upstream regulators of contraction.

In conclusion, by combining scRNA-seq, human cardiac tissue samples, and disease modeling in vitro using isogenic hiPSC-CMs, our findings implicate the UPR pathway in PLN R14del DCM pathogenesis. Furthermore, stimulating the UPR with the small molecule BiX led to functional rescue of the contractility deficit in PLN R14del hiPSC-CMs in vitro in a genotype-specific manner, suggesting UPR as a potential new therapeutic target. Finally, because disease penetrance in PLN R14del DCM is age related,[35] we theorize that the decline in the proteostasis network capacity associated with aging[36] may enhance the propensity of R14del PLN proteins to form aggregates, increasing disease susceptibility. Our study provides important proof of concept that activation of proteostasis mechanisms has a protective effect on PLN R14del-associated DCM in vitro and could be harnessed therapeutically to delay the onset or slow the progression of disease.

Study Limitations

Although patient-specific hiPSC-CMs offer an attractive experimental model, it has certain limitations. Despite the significant progress in the field over the past few years, the hiPSC-CMs are developmentally immature, and their phenotype is more similar to that of human fetal cardiomyocyte. Further studies in relevant animal models of PLN R14del are needed to translate the in vitro findings and to evaluate the therapeutic potential of targeting UPR signaling network in PLN R14del DCM. Nevertheless, our studies demonstrated the potential of using complex physiological models of patient-derived hiPSC-CMs to better understand the mechanisms of PLN R14del DCM pathogenesis toward the development of mechanism-based therapies.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

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