Modeling Secondary Iron Overload Cardiomyopathy with Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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SUMMARY

Excessive iron accumulation in the heart causes iron overload cardiomyopathy (IOC) which initially presents as diastolic dysfunction and arrhythmia but progresses to systolic dysfunction and end-stage heart failure when left untreated. However, the mechanisms of iron-related cardiac injury and how iron accumulates in human cardiomyocytes are not well understood. Herein, using human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), we modeled IOC and screened for drugs to rescue the iron overload phenotypes. Human iPSC-CMs under excess iron exposure recapitulate early-stage IOC, including oxidative stress, arrhythmia, and contractile dysfunction. We found that iron-induced changes in calcium kinetics play a critical role in dysregulation of cardiomyocyte functions. We identified that ebselen, a selective DMT1 inhibitor and antioxidant, could prevent the observed iron overload phenotypes, supporting the role of DMT1 in iron uptake into the human myocardium. These results suggest that ebselen may be a potential preventive and therapeutic agent for treating patients with secondary iron overload.
INTRODUCTION

Hemochromatosis, or iron overload, is a condition in which excess systemic iron accumulation leads to cellular and organ dysfunction and damage. Primary hemochromatosis is an inherited disorder caused by mutations in genes associated with iron homeostasis, such as *HFE, HJV, HAMP, TFR2*, and *SLC40A1* (Gulati et al., 2014; Kremastinos and Farmakis, 2011). Secondary hemochromatosis is caused by excessive iron supply to the body, typically as a result of repeated blood transfusion for treatments of hemoglobinopathies such as thalassemia or sickle cell disease (Farmakis et al., 2017). The resultant systemic iron overload, irrespective of the etiologies, leads to excessive iron deposition in select tissues such as the liver, pancreas, and heart. Iron deposition in the heart manifests clinically as progressive contractile dysfunction and arrhythmia that ultimately leads to end-stage heart failure. Iron overload cardiomyopathy (IOC) is the leading cause of morbidity and mortality in patients with hemochromatosis.

Under normal physiologic conditions, circulating iron exists in a transferrin-bound form that can be transported into cells through transferrin receptors. When transferrin is oversaturated with excessive iron, non-transferrin bound free iron enters the cells from plasma and overloads cytosolic labile iron (Bartnikas et al., 2011). Labile iron has been shown to elicit a variety of cytotoxic effects leading to significant contractile and electrophysiological impairments in the heart (Winterbourn, 1995; Xie et al., 2009). However, the precise effects and mechanisms of iron overload in the human myocardium are not well understood.

Herein, we establish a human *in vitro* model of IOC using induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) and conduct a series of investigations to understand the direct mechanisms of IOC, define the mode of iron entry in human cardiomyocytes, and identify drugs to rescue the IOC phenotypes (Figure 1A).

RESULTS

Labile Iron Accumulates in iPSC-CMs in a Concentration- and Time-Dependent Manner

Human iPSCs generated from three healthy individuals were differentiated into beating cardiomyocytes using a chemically defined Wnt modulation protocol (Burrage et al., 2014). First, to determine the optimal iron concentration to recapitulate iron overload phenotype, we treated iPSC-CMs with increasing concentrations of divalent iron and generated a dose-response curve. Iron uptake was measured by quenching the fluorescence signal of Calcein acetoxymethyl ester (AM) in the presence of intracellular labile iron (Bartnikas et al., 2011). Labile iron has been shown to elicit a variety of cytotoxic effects leading to significant contractile and electrophysiological impairments in the heart (Winterbourn, 1995; Xie et al., 2009). However, the precise effects and mechanisms of iron overload in the human myocardium are not well understood.

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cellular membrane. When comparing cellular iron uptake paced at 2 Hz (120 beats per minute (bpm)) vs without pacing (natural beating rate measured to be ~30–50 bpm), no significant differences in iron uptake were observed (Figure S1D).

**Iron-Overloaded iPSC-CMs Exhibit Significant Contractile Dysfunction**

To assess whether iron overloaded iPSC-CMs recapitulate contractile dysfunction, a key clinical manifestation of IOC, we employed a three-dimensional (3D) scaffold-free cardiac spheroid model (Beauchamp et al., 2015). The generated cardiac spheroids (CSs) were of consistent size (ranging ~300–350 µm) (Figure 1D) and represented a multicellular 3D model (Figure S1E). We first confirmed iron loading into the iPSC-CSs by performing T2-gated MRI (Figure S1F). Consistent with previous clinical MRI studies of iron overload cardiomyopathy (He, 2014), the iPSC-CSs exhibited concentration-dependent and homogenous uptake of the iron. Following iron treatments, we observed significant impairment in both cellular contraction and relaxation, when compared to controls (Figure 1E, Video S1–3). Furthermore, a significant decrease in the beating rates of iPSC-CSs was observed following iron overload (Figure 1F).

**Iron Accumulation Causes Oxidative Stress and Mitochondrial Damage**

Significant oxidative stress has been linked to IOC (Khamseekaew et al., 2017). Similarly, we found a significant, time-dependent increase in intracellular ROS following iron overload in iPSC-CMs (Figure S2A). Next, we examined the mitochondrial-specific ROS production induced by iron overload using the MitoSOX red, a mitochondrial superoxide indicator. We observed a gradual increase in the proportion of MitoSOX-positive iPSC-CMs in both 2D and 3D cultures with increasing iron concentrations (Figures S2B, 1G, and 1H). Similarly, mitochondrial membrane potential as measured by JC-10 staining was significantly reduced in presence of high iron concentration (400 µM) (Figure S2C). Transmission electron microscopy (TEM) images showed hyperpigmentation of mitochondria in iron-treated iPSC-CMs, indicative of mitochondrial iron overload, which was partially reversed by an iron chelator deferoxamine (Figure S2D). Finally, we observed significant lipid peroxidation in iron overloaded iPSC-CMs (Figure S2E), indicating ROS-induced cell membrane damage (Latunde-Dada, 2017). Overall, our data suggest that iron overload induces mitochondrial oxidative stress and cellular damage.

**Iron Overload Causes Arrhythmia and Electrophysiologic Disturbances**

To test whether iron overloaded iPSC-CMs would exhibit arrhythmic phenotypes in vitro, we first stained iPSC-CMs with the calcium indicator Fura-2 AM and assessed beat rate, beat duration, and beat-to-beat intervals. Greater than 60% of iron overloaded iPSC-CMs exhibited arrhythmias, with ~20% showing cessation of beating at 100 µM concentration (Figure S3A). Significant variations in beat-to-beat intervals and beat durations were also observed compared to controls (Figure S3B). Using MEA, we then investigated the electrical defects triggered by iron overload in iPSC-CMs and found a significant, iron concentration-dependent prolongation of field potential duration (FPD) (Figure 2A). Similarly, local extracellular action potential measured by MEA showed a significant prolongation of the action potential duration (APD) upon iron treatment (Figure S3C). Patch clamp recording confirmed a significant prolongation of AP duration by iron overload at the single cell level.
Iron Overload Dysregulates Calcium Kinetics

To explain the observed contractile and electrophysiological phenotypes, we tested whether iron overload influences calcium handling properties of iPSC-CMs. Following 5 days of iron overload at 100 μM, iPSC-CMs exhibited markedly abnormal calcium kinetics with significantly prolonged calcium decay tau (τ) in all three tested cell lines (Figure 2C), suggesting impaired calcium removal kinetics. Changes in the other parameters of calcium handling such as transient amplitude and diastolic calcium, however, did not have consistent trends. We then challenged iPSC-CMs with caffeine to assess the Na⁺/Ca²⁺ exchange (NCX) function as caffeine induces total Ca²⁺ release from the sarcoplasmic reticulum (SR) to the cytoplasm, which is then primarily cleared by NCX. There was no change in caffeine-induced calcium transient amplitude following iron treatment. However, we found that calcium transient decay was consistently and significantly shorter in all iron overloaded iPSC-CMs compared to control iPSC-CMs (Figure 2D), suggesting enhanced calcium extrusion by NCX after iron treatment. Overall, these findings indicate that iron overload (1) impairs the activity of sarco/endoplasmic reticulum calcium ATPase (SERCA) to transport calcium back into the SR as evidenced by prolonged calcium decay and (2) enhances cytosolic calcium removal via NCX as evidenced by shortened caffeine-induced calcium decay.

Iron Overload Alters the Human Cardiomyocyte Transcriptome

To examine transcriptomic changes exerted by iron overload that may explain the observed changes in calcium kinetics, we next performed RNA-sequencing of the three iPSC-CM lines treated with and without iron. Principal component analysis (PCA) showed that cardiomyocytes clustered together based on the iron overload status (Figure 3A). We found that iron overload resulted in 169 upregulated and 150 downregulated gene expression changes (Figure 3B). These trends were also apparent in the generated heatmap (Figure 3C). Furthermore, Gene Ontology (GO) analysis for functional enrichment revealed broad effects on biological processes, molecular functions, and cellular components (Figure 3D), most notably in the cardiovascular system process and development, which indicates a wide-ranging influence of iron overload in iPSC-CMs. When examining the expression of iron-responsive gene products (Figure 3E), we found a significant downregulation of the transferrin receptor 1 (TFRC) and an upregulation of the ferritin light chain (FTL), reflecting the overall iron overloaded state of iPSC-CMs. We next assessed the expression pattern of calcium handling genes in light of the significantly altered calcium kinetics. Interestingly, iron overload led to minimal changes in the expression of calcium handling genes, except for Calsequestrin 2 (CASQ2) known to be involved in calcium storage in SR (Figure 3F). These results suggest that the observed abnormal calcium kinetics were mediated by post-translational modification of calcium handling proteins.
Divalent Metal Transporter 1 Is a Major Iron Entry Channel

We next investigated how free iron enters into cardiomyocytes. Informed by previous studies suggesting the potential roles of L-type calcium channel (LTCC), T-type calcium channel (TTCC), and divalent metal transporter 1 (DMT1) in cardiomyocyte iron uptake (Wijarnpreecha et al., 2015), we investigated the role of these channels in iron uptake by blocking them with respective chemical inhibitors. A known inhibitor of the transferrin-bound iron, an anti-TfR1 antibody, as well as a free iron chelator, deferoxamine (DFO) were used as controls. We first confirmed that all three channels were expressed in iPSC-CMs from the RNA-seq data (Figure S4A). Next, we determined the optimal treatment doses of the inhibitors by obtaining dose-response curves for each inhibitor to identify maximally tolerated doses while maintaining cell viability above 90% (Figure S4B). iPSC-CMs were then co-treated with divalent iron (100 µM) and the respective inhibitors for 5 days to assess iron uptake. As expected, treatments with DFO and anti-TfR1 antibody effectively reduced iron uptake (Figure 4A–C). Among the inhibitors investigated, only ebselen produced a significant reduction in the percentage of iron-positive cells similar to the levels of DFO and anti-TfR1 treatments, implicating an important role of DMT1 in iron entry to the human iPSC-CMs.

Ebselen Rescues Iron-Overload-Induced Phenotypes in Human iPSC-CMs

We next investigated whether ebselen treatment would rescue the observed iron-overload-mediated dysfunction. Indeed, ebselen treatment led to near restoration of calcium decay $\tau$ as well as of FPD (Figures 4D and 4E). Moreover, ebselen treatment decreased ROS production in the iron overloaded iPSC-CMs compared to iron chelator or TfR1 antibody (Figure 4F), suggesting its potential added benefit as an antioxidant. Finally, ebselen treatment improved beating parameters altered by iron (Figures 4G and 4H). Taken together, our data indicate that ebselen, a selective inhibitor of DMT1 and antioxidant, can effectively prevent iron overload cardiotoxicity in human iPSC-CMs.

DISCUSSION

Cardiac iron accumulation causes serious pathological manifestations and mitochondrial dysfunction, leading to IOC (Gordan et al., 2018). Mitochondrial iron overload can cause mitochondrial oxidative stress (Sung et al., 2019), DNA damage (Gao et al., 2009), and membrane depolarization. Mitochondrial reserve plays a critical role in myocardial function as well as in calcium handling (Chang et al., 2016), and our findings indeed show mitochondrial oxidative stress and cellular damage as a contributing factor toward IOC.

To further define the mechanism of contractile dysfunction, we examined calcium kinetics in iron overloaded iPSC-CMs. Our results demonstrate a significant increase in calcium decay, which reflects the impaired SERCA activity in iPSC-CMs that results in decreased clearance of cytosolic calcium. This not only explains the prolonged APD but also diastolic dysfunction, which is typically caused by increased cytosolic calcium retention. Increased expression of $CASQ2$, a marker for calcium storage in SR, also supports impaired calcium clearance. On the other hand, iron overload resulted in significant and consistent decrease in $\tau$ after caffeine treatment, which indicates enhanced activity of NCX to export calcium out
of the cytoplasm and transport sodium into the cytoplasm. This may be a compensatory mechanism to a calcium overloaded state in iron overloaded iPSC-CMs. This net opposing effects of enhanced NCX activity and reduced SERCA activity likely explain in part why diastolic calcium and transient amplitudes remain unaltered. At the same time, enhanced NCX activity induced intracellular sodium overload, which explains the EAD-like occurrences and arrhythmic potential. Interestingly, none of the calcium-handling genes except CASQ2 had significantly altered expression in the RNA-seq analysis, suggesting that the observed abnormal calcium kinetics may be directly mediated by the labile iron.

Thus far, the main therapeutic approach for iron overload has been iron chelation therapy, which aims to deplete the cytosolic iron levels and thereby reduce iron entry. Iron chelators, such as deferoxamine, deferiprone, and deferasirox, directly bind iron in plasma and tissues and induce excretion of excess iron from the body (Fabio et al., 2007). While this approach has been shown to be beneficial, these drugs have limited efficacy of clearing iron in mitochondria (Shvartsman et al., 2007) and is fraught with side effects such as ototoxicity and neurotoxicity, which restricts the long-term use of the drugs (Mobarra et al., 2016).

Our study suggests that ebselen may provide a targeted strategy to prevent iron-induced cardiotoxicity. Ebselen, a selective inhibitor of DMT-1, is a synthetic organoselenium small molecule that mimics glutathione peroxidase with antioxidant and anti-inflammatory effects (Nakamura et al., 2002). In this study, ebselen not only prevented intracellular iron uptake but decreased ROS production of the iron overloaded iPSC-CMs to a much greater extent than iron chelator deferoxamine or Tfr1 antibody, suggesting its dual beneficiary effects. Indeed, treatment with ebselen was sufficient to rescue in vitro iron overload phenotype supporting its potential role as a therapeutic agent. Ebselen has been investigated in treating various neurologic and cardiovascular diseases (Cheng et al., 2019; Jia et al., 2018; Kil et al., 2017) and may also provide a promising therapeutic solution for IOC.

The iPSC technology enables generation of functional human cardiomyocytes and malleable investigation of cardiotoxic effects as induced by iron overload (Burridge et al., 2014; Paik et al., 2020; Takahashi and Yamanaka, 2006). However, immaturity of iPSC-CMs similar to fetal cardiomyocytes may affect their response to stimuli or drugs being tested (Karbassi et al., 2020). Therefore, caution should be practiced when assessing drug response using the iPSC platform.

In summary, we successfully demonstrate an IOC model in a dish using human iPSC-CMs. Our data validate the results from animal studies and confirm increased oxidative stress as a disease mechanism in human cardiomyocytes. Additionally, our data show that iron overloaded iPSC-CMs exhibit an increased burden of arrhythmia and contractile dysfunction, mediated by disturbed calcium kinetics. We identify ebselen, a selective DMT-1 inhibitor and antioxidant, as a potential therapy to rescue the disease phenotype. Our platform will enable future investigations to better elucidate the diverse effects of iron to the human heart and also can serve as an efficient approach to discover and screen for new drugs to rescue iron overload phenotypes.
STAR Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Joseph C. Wu (joewu@stanford.edu).

Materials Availability—Three human iPSC lines were generated from healthy individuals and are available upon request from the Lead Contact.

Data and Code Availability—Original RNA-seq data generated in this study have been deposited to GEO: GSE141678 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141678), titled Transcriptomic analysis of the effect of iron overload in human iPSC-CMs.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human iPSC lines and maintenance—Experiments using human iPSCs were approved by Stanford Research Compliance Office and informed consent was obtained from donors from whom iPSCs were derived. Three human iPSC lines were generated from three healthy individuals by reprogramming with the Sendai virus expressing 4 Yamanaka factors (CytoTune®-iPS Sendai Reprogramming Kit, Invitrogen), as previously described (Churko et al., 2013). For passaging, iPSC culture was dissociated with 0.5 mM EDTA in PBS at 37°C for 5–10 min, and suspended iPSCs were reseeded on Matrigel-coated plates (BD Biosciences, San Jose, CA) at a density of 500 K cells per well in 6-well plates.

METHOD DETAILS

Differentiation of iPSCs into beating cardiomyocytes.—Beating iPSC-CMs were differentiated using chemically defined Wnt modulation protocol as previously described (Burridge et al., 2014). Differentiated iPSC-CMs were maintained in culture until day 30 before conducting all functional and expression analyses.

Fabrication of 3D cardiac spheroids.—Human iPSC-derived cardiomyocytes were dissociated using TripLE Express (Sigma) between day 30 and day 35 and dispensed at a final cell density 1 × 10⁶ cells/mL into sterilized precast agarose replica prepared from silicone molds (Microtissues® Inc). The agarose replica formed has 81 microwells that retain self-assembled scaffold-free cardiac spheroids. The cell suspension added to the molds is allowed to settle into the microwells for two hours at 37°C and 5% CO₂. The spheroids were then maintained in culture with RPMI media supplemented with B27 Supplement (Gibco). After 5 days of iron treatment, brightfield images were taken on Echo Revolve Microscope (Echo Laboratories) at 4X to quantify spheroid size using Image J (NIH). Two to three replicate measurements were pooled per condition (n=40–60).

Iron and inhibitor treatment.—Cells were incubated with various concentrations of ferric ammonium citrate (FAC, Sigma-Aldrich) and 1 mM ascorbic acid (Sigma-Aldrich) to provide iron in a divalent form. To examine the effects of inhibitors blocking LTCC, TTCC,
DMT1, or Tfr1, iPSC-CMs were co-incubated with iron with or without inhibitors for 5 days. Deferoxamine (Sigma-Aldrich, 0 to 20 μM), anti-Tfr1 antibody (R&D system, 0 to 10 μg/ml), verapamil hydrochloride (Sigma-Aldrich, 0 to 100 μM), nifedipine (Sigma-Aldrich, 0 to 5 μM), amlodipine (Sigma-Aldrich, 0 to 200 μM), efonidipine (Sigma-Aldrich, 0 to 100 μM), mibebradil (Sigma-Aldrich, 0 to 70 μM), and Ebselen (Sigma-Aldrich, 0 to 200 μM) were used.

**Iron uptake imaging.**—iPSC-CMs plated on 96-well plates were treated with iron ± drugs for 5 days. Cells were incubated with 1 μM Calcein-AM and Hoechst 33342 (NucBlue Live ReadyProbes Reagent; 2 drops for 1 ml of media) for 30 min at 37°C. Stained cells were washed three times with PBS and fluorescence images were taken and analyzed by Cytation 5 plate reader/imager (BioTek).

**Flow cytometry to assess iron entry.**—iPSC-CMs treated with iron ± drugs were incubated with 0.1 μM Calcein AM and 1 μM EthD-1 in culture medium for 20 min at 37°C. Stained cells were then washed three times with PBS, dissociated in TrypLE solution, and resuspended in PBS supplemented with 10% KSR. Stained cells were analyzed on a FACS ARIA II flow cytometer (BD Biosciences) using the FACS DIVA software and FlowJo v10 software (TreeStar).

**Quantitative plate-based ROS, mitochondrial membrane potential, and cell viability assays.**—Human iPSC-CMs plated on 96-well plates were subjected to plate-based assays after 5 days of iron ± drug treatment using a Cytation 5 plate reader/imager (BioTek). ROS-Glo H2O2 (Promega) was used for ROS detection, JC-10 (Abcam) was used for mitochondrial membrane potential, and Cell Titer Glo (Promega) was used for cell viability assay. All assays were performed according to the manufacturer’s instructions.

**Detection of lipid peroxidation.**—iPSC-CMs were plated on 96-well plates and treated with or without 100 μM iron for 5 days. Cells were incubated in FBS-free medium with 10 μM C11-BODIPY (581/591) and Hoechst 33342 (NucBlue Live ReadyProbes Reagent; 2 drops for 1 ml of media) for 30 min at 37°C. Stained cells were washed three times with PBS and the fluorescence images were taken using Cytation 5 (BioTek).

**High-resolution T2-weighted magnetic resonance imaging (MRI).**—Three groups of iPSC-derived cardiac spheroids treated with different iron concentrations (0 μM, 100 μM, and 400 μM) were held in three silicone-casted culture molds. Before imaging, these culture molds were steadily held into 15 mL polypropylene centrifuge tubes immersed in PBS (pH 7.4). After that, they were placed in a Varian millipede coil and scanned by a 7T Bruker horizontal bore scanner (Bruker, Billerica, MA) with a shielded gradient system (600 mT/m). These cardiac spheroids were imaged with a fast spin-echo sequence for T2-weighted imaging: echo time (TE) 32 ms, repetition time (TR) 2200 ms, number of signal average 5, slice thickness 0.5 mm, no slice separation, field of view (FOV) 25×25 mm², and in-plane resolution of 49×49 μm². Post image acquisitions, the region of interests of all cardiac spheroids were manually segmented on MR images by an experienced expert using an image analysis software Segment (http://medviso.com/). The MR intensity of each cardiac spheroid was calculated as the mean value of its segmented region of interests.
Transmission electron microscopy.—iPSC-CMs were re-suspended in 20% BSA in PBS, placed into a 200 μM deep hat, and high pressure-frozen using a Leica EMpact2. Frozen samples were then freeze-substituted in 1% osmium tetroxide and 0.1% uranyl acetate in acetone using Kent McDonald’s quick method in a cold block, allowed to warm in a styrofoam block for 3 hours to 0°C and moved to a hood for 30 min, held for another 12 hours and then warmed to 0°C in 5 hours @ 5°C/hr, and held for a final 12 hours. The samples were washed in 2X in acetone and 1X in propylene oxide (PO) for 15 min each. Samples are infiltrated with EMbed-812 resin (EMS Cat#14120) mixed 1:2, 1:1, and 2:1 with PO for 2 hours each, leaving samples in a 2:1 resin-to-PO ratio overnight rotating at RT in the hood. The samples are then placed into EMbed-812 for 2 to 4 hours, placed into TAAB capsules w/labels and fresh resin, orientated with specimen carrier/cells (if still in hat) facing up, and placed into 65°C oven overnight. Sections were taken between 75 and 90 nm, picked up on formvar/carbon-coated 100 mesh Cu grids, then contrast-stained for 30 seconds in 3.5% uracetate in 50% acetone followed by staining in 0.2% lead citrate for 3 to 4 min. Sections were observed in the JEOL JEM-1400 at 120kV and photos were taken using a Gatan Orius 2k × 4k digital camera.

Fura-2 ratiometric calcium imaging.—iPSC-CMs were dissociated by TrypLE and re-seeded in Matrigel (BD Bioscience) pre-coated 24×50 mm coverslips (Warner Instrument). After a 7-day recovery period, cells were treated with iron ± drugs for 5 days. Cells were loaded with 5 µM Fura-2 acetoxymethyl ester (AM) with 0.1% F-127 for 10 min at RT in Tyrode’s solution. iPSC-CMs were paced at 0.5 Hz. In some experiments, pacing was stopped after 8 secs and 25 mM caffeine was applied to induce total calcium release from SR. Single-cell calcium handling was observed with a Nikon Eclipse Ti-E inverted microscope mounted with 40× oil immersion objective (0.95 NA). A Lambda DG-4 ultra-high-speed wavelength switching light source (Sutter Instrument) was used to excite Fura-2 at 340 nm and 380 nm wavelength. The emission signals at >510 nm were collected with an iXon Ultra 897 EMCCD (Andor) as a high-frame rate video (512*512, 50 fps). Calcium transient was calculated as 340/380 ratio. Raw imaging data were analyzed with the Greensmith method.

Contractility motion vector analysis.—Cardiac spheroid contractility was assessed as previously described (Hayakawa et al., 2012). Briefly, the contraction of cardiac spheroids was recorded under high resolution motion capture tracking using the SI8000 Live Cell Motion Imaging System (Sony Corporation) before and after 5 days of iron ± drug treatment. During data collection, cells were maintained under controlled humidified conditions at 37°C with 5% CO₂ and 95% air in a stage-top microscope incubator (Tokai Hit). Functional parameters were assessed from the averaged contraction-relaxation waveforms from 10-sec recordings, using the SI8000C Analyzer software.

Patch clamp recordings.—Electrophysiological properties of each iPSC-CM line were recorded using an EPC-10 patch clamp amplifier (HEKA, Germany). Monolayers from lines 1, 2, and 3 were enzymatically dissociated in 35 mm petri dishes coated with ESC-qualified Matrigel (Corning), then treated with either iron, deferoxamine (DFO), transferrin receptor 1 antibody (anti-TfR1), Ebselen, or vehicle as a control for 5 days. All recordings were
performed at 37°C in a chamber (Warner instrument, USA) mounted on an inverted microscope (Nikon, Japan). Thin wall glass filaments were pooled to obtain 2.5–4 MΩ pipettes using a micropipette puller (Sutter Instrument, USA). For current clamp, action potentials (APs) were recorded in perforated-patch mode to preserve membrane integrity on single iPSC-CMs. Spontaneous APs were recorded before various pacing cycles were applied: 0.5, 1, and 2 Hz.

**RNA-sequencing.**—Total RNA of iPSC-CMs was isolated using Qiagen miRNeasy Mini Kit (Qiagen Sciences, Inc, Germantown, MD). Sequencing libraries were generated using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina. The raw 76 bp paired-end RNA-seq reads (56 minion reads per sample on average) sequenced by Illumina HiSeq 2000 were first trimmed for quality control by TrimGalore version 0.4.2 ([https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)) to exclude adapter sequences and bases with Phred scores less than 20, reflecting a low probability of base-calling errors (p<1%), equivalent to >99% accuracy. On average, 98.84% bases passed the filtering. The trimmed RNA-seq reads were then mapped to hg38 using STAR software (Dobin et al., 2013) with ENCODE options. Bam file contains reads mapped to GENCOD (Harrow et al., 2012) (hg38, version 25) annotated transcriptome regions were used for estimating gene expression by DESeq2 in R (Love et al., 2014). All data are deposited in (GEO:GSE141678)

**Detection of differentially expressed genes.**—We used DESeq2 in R to detect differentially expressed genes between groups. To control the confounding effects of the cell line, we set the full model as full = ~treatment + line and the reduced model as reduce = ~line. Genes with adjusted p-value (false discovery rate, FDR) <5% and |log2FC| ≥1 are defined as DEGs.

**Functional enrichment analysis.**—Functional enrichment analyses of differentially expressed genes were performed using the Bioconductor package “GeneAnswers” (Feng et al., 2010) in R. Functional annotations were from Gene Ontology (Ashburner et al., 2000). Functional annotated terms with a false discovery rate (FDR) <5% were considered as significantly enriched. P values before multiple test corrections for each functional annotated term were calculated from the hypergeometric test as following:

\[
p = 1 - \sum_{k=0}^{m-1} \binom{M}{k} \binom{N-M}{n-k} \binom{N}{n}^{-1}
\]

Where N is the total number of genes with functional annotation across the genome; n is the number of differentially expressed genes in N; M is the number of genes annotated for a particular functional annotated term across the genome; and m is the number of differentially expressed genes annotated for the particular functional annotated term. Bubble plots and chord plots were generated using “GOplot” package in R (Walter et al., 2015) to visualize the functional enrichments. Z scores in bubble plots were calculated as following:
\[ z_{\text{score}} = \frac{\text{up} - \text{down}}{\sqrt{\text{count}}} \]

Where count is the total gene number, and up and down are the number of up-regulated or downregulated differentially expressed genes, respectively.

**Cellular pacing and field potential recordings.**—The field potential recordings as well as cellular pacing were performed using microelectrode array technology (MEA) from Axion Biosystems. First, iPSC-CMs were enzymatically dissociated and then plated on 48-well plates (Axion Biosystems) that were previously coated with ESC-qualified Matrigel (Corning). MEA experiments were achieved at day 30 after cardiac differentiation, with both baseline and iron overloaded +/- inhibitor conditions being recorded at 37°C in a 5% CO₂ environment. Data acquisition was performed using the Axion Biosystem software, data analysis was completed using the CIPA software, and final FPD (Field Potential Duration) values were obtained by subtracting the treatment data from the baseline and displayed in % change.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis.**—For statistical analysis, if data were normally distributed, a Student’s t-test was used to compare the differences between two data sets. For comparisons among multiple groups, one-way or two-way ANOVA was used, where appropriate, and the Holm-Sidak post-hoc test was used for all pairwise comparisons, depending on the properties of the data sets. A P value <0.05 was considered to be statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Unless otherwise stated, all data in bar graphs were presented as mean ± standard error mean.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The effects of labile iron accumulation in iPSC-CMs.

Human iPSC-CMs from 3 healthy subjects were treated with divalent iron (100 μM) for 5 days. (A) Overall schematic of the experimental design. (B) Representative flow cytometry scatter plots showing iron overload in iPSC-CMs. Cells were labeled with Calcein-AM (for staining iron-negative cells) and EthD-1 (for staining dead cells) prior to analysis by flow cytometry. (C) The percentage of iron-positive-cells after iron treatment. (D) Size distribution of 3D iPSC-derived cardiac spheroids. (E) Contractile kinetics of the spheroids treated with divalent iron (0 μM, 100 μM, or 400 μM). Max contraction velocity (left) and max relaxation velocity (right) were significantly reduced by iron overload. (F) Beating rates of the spheroids significantly decreased with iron overload. (G) Mitochondrial ROS assessed using mitochondrial superoxide indicator, MitoSOX Red, showed a gradual increase in the proportion of MitoSOX-positive cardiomyocytes with increasing iron concentrations. (H) The percentage of MitoSOX-positive cells after iron treatments. All results are representative of at least three independent experiments. Graphs show mean ± SEM. (*p<0.05, **p<0.01).
Figure 2. Electrophysiological and calcium handling effects of iron overload in iPSC-CMs.

(A) Multi-electrode array recordings of iron-overloaded iPSC-CMs show prolongation of field potential duration (FPD) with increasing iron concentration (0 μM, 100 μM, and 400 μM). (B) Patch clamp recordings of iron-overloaded iPSC-CMs show increase in action potential duration (APD) with increasing iron concentration. (C) Calcium kinetics study using Fura-2 AM shows significantly prolonged decay tau following iron treatment in all three cell lines (n=50, each). (D) Calcium kinetics following caffeine challenge shows significantly shortened decay tau following iron treatment in all three cell lines (n=20, each). (*p<0.05, **p<0.01, ***p<0.001).
Figure 3. Transcriptomic signatures of iron-overloaded iPSC-CMs.

(A) Principal component analysis of gene expressions across iPSC-CMs ± iron treatments show clustering of the samples based on the iron overload status. (B) Volcano plot of gene expression changes. The x-axis specifies the $\log_2$ fold-changes (FC) and the y-axis specifies the $-\log_{10}$ false discovery rates (FDR). Red dots are the genes with $(|\log_2 FC| \geq 1$ and FDR $<5\%)$; blue dots are the genes with $(|\log_2 FC| <1$ and FDR $<5\%)$; orange dots are the genes with $(|\log_2 FC| \geq 1$ and FDR $>5\%)$; and grey dots are the genes with $(|\log_2 FC| <1$ and FDR $>5\%)$. Pairwise comparison of differentially expressed genes identified 169 significantly upregulated genes (2-fold increase) and 150 significantly downregulated genes (<0.5-fold decrease) following the iron treatment. (C) Heatmap of differentially expressed genes revealed distinct expression patterns based on iron overload status. (D) Bubble plot of pathway enrichment via Gene Ontology (GO) analysis revealed effects on multiple biological processes, molecular functions, and cellular components. (E) The expression of iron-responsive gene products showed significant downregulation of transferrin receptor (TFRC) and upregulation of ferritin light chain (FTL). (F) The expression of calcium handling genes did not reveal significant alterations following iron treatment except for Calsequestrin 2 (CASQ2).
Figure 4. The mechanism of labile iron accumulation in iPSC-CMs and restoration of iron-overload phenotypes with ebselen, a DMT1 inhibitor.

(A–C) To identify the channel by which labile iron enters into cardiomyocytes, iPSC-CMs were treated with increasing doses of iron-entry channel inhibitors such as verapamil (1 μM), amlodipine (1 μM), and nifedipine (3 μM) to block L-type calcium channel (LTCC); efonidipine (0.4 μM) and mibefradil (0.2 μM) to block T-type calcium channel (TTCC); and ebselen (20 μM) to block divalent metal transporter 1 (DMT1). Deferoxamine (DFO, 20 μM) and transferrin receptor 1 antibody (anti-TfR1, 5 μg/mL) were used as positive controls preventing iron entry into iPSC-CMs. Cells were co-stained with Calcein-AM (for staining iron-negative cells) and EthD-1 (for cell viability), prior to flow cytometry analysis. (A) Representative flow cytometry scatterplots showing that only ebselen resulted in a significant reduction in iron-positive cells among the tested channel inhibitors. (B) The percentage of dead cells after iron +/− inhibitor treatments. (C) The percentage of iron-positive cells after iron +/− inhibitor treatments. (D–F) Ebselen rescues iron overload in
vitro phenotypes. All comparisons were made to Fe2+ 100-mM-treated iPSC-CMs. Ebselen treatment (D) normalized decay tau in calcium kinetics assays, (E) led to near restoration of FPD in MEA assays, and (F) markedly reduced ROS levels. (G and H) Ebselen treatment (G) restored the abnormal beating rate (beats per minute) of the iPSC-CMs and (H) significantly reduced the beating rate variation to levels comparable to the positive controls with DFO and anti-TfR1. All results are representative of at least three independent experiments. Mean ± SEM is presented. p values were obtained from Student’s t test (*p < 0.05, **p < 0.01, ***p < 0.001).
# KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-Cardiac Troponin T antibody | Abcam | Cat# ab45932; RRID:AB_956386 |
| Human TIR (Transferrin R) Antibody | R and D Systems | Cat# AF2474, RRID:AB_416601 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Corning™ matrigel™ membrane matrix | Fisher Scientific | CB-40234 |
| Calcein, AM, cell-permeant dye | Invitrogen | C3100MP |
| Verapamil hydrochloride | Sigma Aldrich | V4629–1G |
| Ebselen cysteine modifier | Sigma Aldrich | E3520–25MG |
| L-Ascorbic acid BioXtra, ≥99.0%, crystalline | Sigma Aldrich | A5960–25G |
| Ammonium iron(III) citrate | Sigma Aldrich | F5879 |
| Amlopidine besylate | Sigma Aldrich | A5605–10MG |
| Nifedipine | Sigma Aldrich | N7634–1G |
| Mitoxo Red | Life Technologies | M36008 |
| NucBlue® Live ReadyProbes® Reagent | Life Technologies | R37605 |
| BODIPY® 581/591 C11 | Thermo Fisher | D-3861 |
| Fura-2, AM, cell permeant | Molecular Probes | F-1221 |
| **Critical Commercial Assays** |        |            |
| CellTiter-Glo 2.0 Promega G9242 | Fisher/Promega | PRG9242 |
| ROS-Glo™ H2O2 Assay Promega G8820 | Fisher/Promega | PR-G8820 |
| JC-10 | AAT Bioquest | 22800 |
| **Deposited Data** |        |            |
| Raw and analyzed data | This paper | GEO: GSE141678 |
| **Experimental Models: Cell Lines** |        |            |
| Human induced pluripotent stem cell control lines | Stanford Cardiovascular Institute biobank | SCVI 273, 116, 114 |
| **Software and Algorithms** |        |            |
| ImageJ | NIH | https://imagej.nih.gov/ij/ |
| FlowJo v10 software | FLOWJO | https://www.flowjo.com/solutions/flowjo/downloads |
| Segment | MEDVISO | http://medviso.com |
| SI8000C Analyzer | Sony biotechnology | Cardio Model Software |
| STAR | Dobin et al., 2013 | https://github.com/alexdobin/STAR |
| GeneAnswers | Feng et al., 2010 | R package |
| **Other** |        |            |
| MicroTissues® 3D Petri Dish® micro-mold spheroids | MilliporeSigma | Z764051–6EA |
| CytoView MEA 48 | Axion Biosystems | M768-0MEA-48B |