New Methods in Cardiovascular Biology

Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes as an In Vitro Model for Coxsackievirus B3–Induced Myocarditis and Antiviral Drug Screening Platform

Arun Sharma, Caleb Marceau, Ryoko Hamaguchi, Paul W. Burridge, Kuppusamy Rajarajan, Jared M. Churko, Haodi Wu, Karim I. Sallam, Elena Matsa, Anthony C. Sturzu, Yonglu Che, Antje Ebert, Sebastian Diecke, Ping Liang, Kristy Red-Horse, Jan E. Carette, Sean M. Wu, Joseph C. Wu

Rationale: Viral myocarditis is a life-threatening illness that may lead to heart failure or cardiac arrhythmias. A major causative agent for viral myocarditis is the B3 strain of coxsackievirus, a positive-sense RNA enterovirus. However, human cardiac tissues are difficult to procure in sufficient enough quantities for studying the mechanisms of cardiac-specific viral infection.

Objective: This study examined whether human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) could be used to model the pathogenic processes of coxsackievirus-induced viral myocarditis and to screen antiviral therapeutics for efficacy.

Methods and Results: hiPSC-CMs were infected with a luciferase-expressing coxsackievirus B3 strain (CVB3-Luc). Brightfield microscopy, immunofluorescence, and calcium imaging were used to characterize virally infected hiPSC-CMs for alterations in cellular morphology and calcium handling. Viral proliferation in hiPSC-CMs was quantified using bioluminescence imaging. Antiviral compounds including interferon β1, ribavirin, pyrrolidine dithiocarbamate, and fluoxetine were tested for their capacity to abrogate CVB3-Luc proliferation in hiPSC-CMs in vitro. The ability of these compounds to reduce CVB3-Luc proliferation in hiPSC-CMs was consistent with reported drug effects in previous studies. Mechanistic analyses via gene expression profiling of hiPSC-CMs infected with CVB3-Luc revealed an activation of viral RNA and protein clearance pathways after interferon β1 treatment.

Conclusions: This study demonstrates that hiPSC-CMs express the coxsackievirus and adenovirus receptor, are susceptible to coxsackievirus infection, and can be used to predict antiviral drug efficacy. Our results suggest that the hiPSC-CM/CVB3-Luc assay is a sensitive platform that can screen novel antiviral therapeutics for their effectiveness in a high-throughput fashion. (Circ Res. 2014;115:556-566.)

Key Words: myocarditis ■ myocytes, cardiac ■ stem cells

Myocarditis is characterized by myocardial inflammation that can progress to dilated cardiomyopathy and heart failure.¹² Upwards of 20% of all sudden cardiac deaths in young adults may be caused by myocarditis, which arises via systemic bacterial or viral infections.³⁴ Viral myocarditis remains a difficult disease to diagnose definitively, partly because the preferred diagnostic method is invasive myocardial biopsy.⁵ Group B coxsackieviruses are single-stranded, positive-sense RNA viruses belonging to the Enterovirus genus and may be implicated in 30% to 50% of all myocarditis cases.⁶⁷ The coxsackievirus B3 (CVB3) strain has been the most commonly used strain in animal and cellular models of viral myocarditis.⁸ CVB3 proliferates rapidly within human cardiomyocytes, entering the cell via a transmembrane coxsackievirus and adenovirus receptor (CAR) that is differentially

Editorials, see p 537, 540
In This Issue, see p 533

Original received February 25, 2014; revision received July 8, 2014; accepted July 11, 2014. In June 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15 days.


The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.115.303810/-/DC1.

Correspondence to Joseph C. Wu, MD, PhD, or Sean M. Wu, MD, PhD, Lorry I. Lokey Stem Cell Research Building, 265 Campus Dr, Rm G1120B, Stanford, CA 94305–3454. E-mail jcuwu@stanford.edu or smwu@stanford.edu

© 2014 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.115.303810

556
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAR</td>
<td>coxsackievirus and adenovirus receptor</td>
</tr>
<tr>
<td>CVB3</td>
<td>coxsackievirus B3</td>
</tr>
<tr>
<td>hiPSC-CM</td>
<td>human induced pluripotent stem cell–derived cardiomyocyte</td>
</tr>
<tr>
<td>IFNβ1</td>
<td>interferon β1</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>PDTC</td>
<td>pyrroline dithiocarbamate</td>
</tr>
</tbody>
</table>

expressed on various cell types, is highly expressed in cardiomyocytes, and is essential for mammalian cardiogenesis.\textsuperscript{5–11} CAR also functions as an intercellular adhesion molecule and associates with extracellular matrix glycoproteins such as laminin and fibronectin.\textsuperscript{12} Differential expression of CAR may determine tissue-specific susceptibility to CVB3 infection, and cardiac tissues highly express CAR during development and in dilated cardiomyopathy.\textsuperscript{13–16} After internalization in primary human cardiomyocytes, CVB3 can induce viral cytopathogenic effect and myofiber necrosis within hours.\textsuperscript{10} Given the life-threatening consequences of cardiac viral infection, it is critical to further elucidate the mechanisms of CVB3-induced viral myocarditis.

Obtaining primary human cardiac tissues for research purposes requires invasive surgical procedures. Thus, these tissues are scarce and cannot be cultured easily in vitro, limiting their utility for studying cardiac disease mechanisms.\textsuperscript{17} Although animal models can partially recapitulate human cardiovascular disease phenotypes, they exhibit interspecies differences in heart rate, electrophysiology, and cardiogenesis.\textsuperscript{18} However, with the advent of human induced pluripotent stem cells (hiPSCs), difficult-to-procure cell types such as human cardiomyocytes can now be mass-produced in vitro.\textsuperscript{19,20} These hiPSC-derived cardiomyocytes (hiPSC-CMs) could be used to investigate the pathophysiology and molecular mechanisms of acquired cardiac disorders such as viral myocarditis. Although other cell types such as HEK293T and HeLa have been used to study coxsackievirus infection, these noncardiac cells are not ideal for modeling cardiomyocyte infection in viral myocarditis.\textsuperscript{21,22} Other cardiac cells such as HL-1 mouse atrial tumor cells are immortalized, proliferative cells that are not reflective of adult human cardiomyocytes.\textsuperscript{23} Thus, hiPSC-CMs are ideal cells for studying the mechanisms of coxsackievirus-induced viral myocarditis because they are nonimmortalized human cardiomyocytes that express relevant ion channels and sarcemeric proteins found in adult human cardiomyocytes.\textsuperscript{19,20} Because there are limited therapeutic options for eliminating coxsackievirus-induced infections, hiPSC-CMs could provide a novel platform for screening compounds aiming to treat diseases such as viral myocarditis that are caused by coxsackievirus infection.\textsuperscript{24}

Here, we derived hiPSC-CMs to model CVB3-induced viral myocarditis. We studied CAR expression using immunofluorescence and quantitative gene expression assays and determined that, like primary cardiomyocytes, hiPSC-CMs express CAR and are susceptible to coxsackievirus infection. We also used brightfield microscopy, immunofluorescence, and calcium imaging to characterize alterations in hiPSC-CM structure and function because of CVB3 infection. Finally, we used a luciferase-expressing CVB3 strain (CVB3-Luc) to quantify viral replication on hiPSC-CMs. This study represents the first time that hiPSC-CMs have been used to quantitatively assess the efficacy of antiviral compounds in reducing coxsackievirus proliferation on human cardiomyocytes, thus providing a new platform for screening novel antiviral therapeutics.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Differentiation of hiPSC-CMs From hiPSCs**

Lentiviral reprogramming was used to generate 3 hiPSC lines from skin fibroblasts of 3 healthy individuals in a 7-member family cohort.\textsuperscript{25} An additional 3 hiPSC lines were generated with a previously published Sendai virus reprogramming protocol using peripheral blood mononuclear cells from 3 healthy individuals.\textsuperscript{26} These 6 hiPSC lines were differentiated into hiPSC-CMs using a 2-dimensional monolayer differentiation protocol and were maintained in a 5% CO\textsubscript{2}/air environment as previously published.\textsuperscript{25,27} Briefly, hiPSC colonies were dissociated with 0.5 mmol/L EDTA into single-cell suspension and resuspended in E8 media (Life Technologies) containing 10 mmol/L Rho-associated protein kinase inhibitor (Sigma). Approximately 100,000 cells were replated into 6-well dishes precoated with Matrigel (BD Biosciences). Next, hiPSC monolayers were cultured to 85% cell confluency. Cells were then treated for 2 days with 6 mmol/L CHIR99021 (Selleck Chemicals) in RPMI plus B27 supplement without insulin to activate Wnt signaling and induce mesodermal differentiation. On day 2, cells were plated on RPMI plus B27 without insulin and CHIR99021. On days 3 to 4, cells were treated with 5 mmol/L IWR-1 (Sigma) to inhibit Wnt pathway signaling and induce cardiogenesis. On days 5 to 6, cells were removed from IWR-1 treatment and placed on RPMI plus B27 without insulin. From day 7 onwards, cells were plated on RPMI plus B27 with insulin until beating was observed. At this point, cells were glucose-starved for 3 days with RPMI (no glucose) plus B27 with insulin to purify hiPSC-CMs, because cardiomyocytes can selectively metabolize fatty acids as a source of cellular energy.\textsuperscript{28} After purification, cells were cultured in RPMI plus B27 with insulin. When replating hiPSC-CMs for downstream use, cells were dissociated with 0.25% trypsin EDTA (Life Technologies) into a single-cell suspension and seeded on Matrigel-coated plates.

**CVB3-Luc Infections and Antiviral Treatments**

Stocks from a previously published CVB3-Luc strain expressing Renilla luciferase were stored at −80°C until needed.\textsuperscript{29} Interferon β1 (IFNβ1; Life Technologies), ribavirin (MP Biochemicals), pyrroline dithiocarbamate (PDTC; Sigma), and fluoxetine (Sigma) stocks were dissolved in water. Before CVB3-Luc infection, day 30 to 35 postdifferentiation hiPSC-CMs were pretreated with antiviral compounds for 12 hours unless noted otherwise.

**Bioluminescence Imaging**

Day 30 to 35 postdifferentiation hiPSC-CMs were plated in RPMI plus B27 with insulin on Matrigel at a density of 40,000 cells per well of a 96-well plate. At the time of CVB3-Luc infection, 6 mmol/L Enduren, an extended-duration coelenterazine (Promega), was added. After infection, bioluminescence imaging was conducted using an Xenogen IVIS 100 Imaging System. Living Image software (Perkin Elmer) was used for image analysis.

**Ca\textsuperscript{2+} Imaging**

Dissociated day 30 to 35 postdifferentiation hiPSC-CMs were reseeded in Matrigel-coated 8-well Laboratory Tek II chambers (Nalge Nunc International) and were treated with 5 mmol/L Fluo-4 AM and 0.02% Pluronic F-127 (Molecular Probes) in Tyrode solution for 15 minutes at 37°C. Cells were washed with Tyrode solution afterward. Ca\textsuperscript{2+} imaging was conducted using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss AG) and analyzed using Zen imaging software.
Spontaneous Ca²⁺ transients were obtained at 37°C using a single-cell line scan mode.

**Cell Metabolism and Viability Assays**

WST-1 reagent (Abcam) was used to determine hiPSC-CM metabolism and viability after antiviral treatment. After 48-hour treatment with antiviral compounds, 10 μL of WST-1 reagent was added to 100 μL RPMI plus B27 with insulin on day 30 to 35 postdifferentiation hiPSC-CMs. After 24 hours, a microplate reader (Promega) was used to quantify conversion of tetrazolium salt WST-1 into formazan dye at 420 to 480 nm absorbance. Absorbance reading correlated directly with cell viability.

**Gene Expression and Immunocytochemistry**

For quantitative reverse transcriptase polymerase chain reaction, RNA was extracted with the miRNeasy kit (Qiagen). Complementary DNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and real-time polymerase chain reaction was conducted on an Applied Biosystems 7900HT Fast Real-Time PCR System. Primers are listed in Online Table I. For additional gene expression analysis, a GeneChip Human Gene 2.0 ST DNA Microarray was used (Affymetrix). Immunostaining was performed according to previous protocols. Imaging was performed using a DMIL–LED microscope (Leica Microsystems) or a Zeiss LSM 510Meta confocal microscope (Carl Zeiss AG) using Zen imaging software.

**Statistical Methods**

Data are presented as mean±SEM. Comparisons were conducted via Student t test with significant differences defined by P<0.05. For microarray results, multiple P value comparisons were made using a 1-way between-subjects ANOVA (P<0.05) using Affymetrix Transcriptome Analysis Console 2.0 software. Microarray data were deposited under National Institutes of Health Gene Expression Omnibus (GSE57781).

**Results**

Expression of Cardiomyocyte Markers in hiPSC-CMs

Undifferentiated hiPSCs expressed standard pluripotency markers as shown previously (Online Figure I). We produced hiPSC-CMs from hiPSCs using a monolayer-based cardiac differentiation protocol. We achieved upwards of 90% differentiation efficiency and generated spontaneously contracting sheets of hiPSC-CMs (Online Movie I). Next, hiPSC-CMs were purified via glucose deprivation and replated for downstream analyses (Figure 1A). Cells exhibited regular beating patterns after replating (Online Movie II).

**Figure 1.** Human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) express intracellular sarcomeric proteins and coxsackievirus and adenovirus receptor (CAR) at cell–cell junctions. A, Flow chart illustrating study design. Skin fibroblast samples obtained from 3 healthy individuals in a 7-member patient family cohort were reprogrammed using lentiviral vectors expressing Oct4, Klf, Sox2, c-Myc (OKSM). Peripheral blood mononuclear cells were also isolated from 3 additional healthy individuals and reprogrammed using a Sendai virus vector expressing OKSM. Subsequent hiPSC colonies were differentiated into hiPSC-CMs. Downstream analyses such as immunofluorescence, calcium imaging, and antiviral drug efficacy testing were then conducted on hiPSC-CMs infected with luciferase-expressing coxsackievirus B3 strain (CVB3-Luc). B, Confocal microscopy images of single hiPSC-CMs demonstrates the presence of sarcomeric proteins such as cardiac troponin T (cTnT) and α-actinin. C, Confocal microscopy demonstrates the presence of CAR at hiPSC-CM intercellular junctions (arrow). D, Quantitative reverse transcriptase polymerase chain reaction of hiPSC-CMs at d 0 and 15 after cardiac differentiation reveals a decrease in POU5F1 expression (left), an increase in myosin heavy chain 6α (MYH6) expression (middle), and a decrease in CXADR expression (right). **P<0.01; ****P<0.0001.
Using immunofluorescence, we confirmed that hiPSC-CMs express cardiac-specific sarcomeric proteins. Expression of cardiac troponin T showed a striated pattern intercalated with α-actinin expression along sarcomeric Z-lines (Figure 1B).

Expression of CAR
Immunofluorescence revealed that hiPSC-CMs expressed the CAR at cell–cell junctions (Figure 1C). Nuclear stain for CAR in CMs has been previously reported and may represent antibody cross-reactivity with a nuclear antigen.30 CAR is also expressed in hiPSCs, suggesting that both hiPSCs and hiPSC-CMs are susceptible to coxsackievirus infection (Online Figures I and II). In HL-1 cells, CAR is also expressed at points of cell–cell contact (Online Figure III). We used quantitative reverse transcriptase polymerase chain reaction to confirm significant downregulation of pluripotency gene expression (POU5F1) and significant upregulation of cardiac-specific structural gene expression (myosin heavy chain 6α) in hiPSC-CMs after 15 days of cardiac differentiation (Figure 1D). We also observed ≈2-fold reduction in the expression of CXADR, encoding for CAR, after 15 days of cardiac differentiation (Figure 1D). CXADR expression in hiPSC-CMs is 30-fold less than that of primary adult human left ventricular myocardium sample (Online Figure IV). However, CXADR expression in hiPSC-CMs is 10-fold higher than in HL-1 mouse cardiac cells (Online Figure IV). These results demonstrate that hiPSC-CMs express CAR along with cardiac-specific markers.

Characterization of hiPSC-CMs Infected With CVB3-Luc
Purified hiPSC-CMs were infected with a B3 strain of coxsackievirus expressing Renilla luciferase (CVB3-Luc). CVB3-Luc gene expression strongly correlated to luciferase luminescence in infected hiPSC-CMs, suggesting that luciferase could be used as a direct measure for CVB3-Luc proliferation (Online Figure V). At multiplicity of infection (MOI) 5, virally induced cytopathic effect appeared at 6 to 8 hours postinfection, corresponding to the completion of the CVB3 replication cycle.31 We did not observe a difference in time to cytopathic effect onset among our 6 hiPSC-CM lines at CVB3-Luc MOI 5 (Online Figure VI). Complete cell detachment was apparent at 24 hours postinfection (Figure 2A). Starting at 6 hours postinfection with CVB3-Luc MOI 5, cells displayed irregular beating patterns that became increasingly erratic over time, culminating in the eventual cessation of beating after ≈12 hours of infection (Online Movies III and IV). Onset of cytopathic effect in a purified population of hiPSC-CMs corresponded to increased expression of VP1, a component of the viral capsid (Figure 2B).31 Notably, hiPSCs

Figure 2. Human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) are susceptible to infection by luciferase-expressing coxsackievirus B3 strain (CVB3-Luc) and display irregular intracellular calcium-handling phenotypes during infection. A, Brightfield images of hiPSC-CMs infected with CVB3-Luc (multiplicity of infection [MOI] 5) show the progression of cellular cytopathic effect because of viral infection >24 h. B, Immunofluorescence images of hiPSC-CMs infected with CVB3-Luc (MOI 5) illustrate increasing VP1 expression in a homogeneous population of cardiac troponin T (cTnT)–positive hiPSC-CMs >8 h after infection. C, Immunofluorescence images of a heterogeneous, differentiated cell population illustrates VP1 expression in cTnT–hiPSC-CMs (left, middle), but not in non-CM, α-smooth muscle actin (α-SMA)–positive mesenchymal cells (right) 7 h after infection with CVB3-Luc (MOI 5). D, Single-cell Ca2+ imaging of uninfected hiPSC-CMs and hiPSC-CMs 7 h after infection with CVB3-Luc (MOI 5). *P<0.05; **P<0.01
were also susceptible to CVB3-Luc infection and displayed an increase in VP1 expression after infection (Online Figure VII). Only a small proportion of HL-1 cells in a homogenous population expressed VP1 after CVB3 infection, as described previously (Online Figure III). In a heterogeneous unpurified population of hiPSC-CMs after a low-efficiency cardiac differentiation, cardiac troponin T–positive hiPSC-CMs were more susceptible to CVB3-Luc infection compared with noncardiomyocyte, α-smooth muscle actin–positive mesenchymal cells (Figure 2C). Calcium imaging of cells (n=12) infected with CVB3-Luc at MOI 5 for 7 hours showed a significant reduction in beating rate and increases in calcium transient duration, time to transient peak, and standard deviation of transient intervals, suggesting that CVB3-Luc infection results in disrupted intracellular calcium handling in hiPSC-CMs (Figure 2D). Taken together, these results suggest that hiPSC-CMs are highly susceptible to coxsackievirus infection and that viral infection causes detrimental alterations in hiPSC-CM structure and function.

Quantification of CVB3-Luc Proliferation on hiPSC-CMs
We next used bioluminescence imaging to quantify CVB3-Luc proliferation on hiPSC-CMs. Purified hiPSC-CMs were infected with decreasing MOI of CVB3-Luc in the presence of Enduren (Figure 3A). CVB3-Luc proliferation was quantified based on bioluminescence intensity (radiance), corresponding to the amount of luciferase, and thus virus, being produced. At MOI 5, bioluminescence signal spiked at 6 hours postinfection, corresponding to the completion of the first viral replication cycle. Signal intensity increased until 12 hours postinfection, at which point bioluminescence diminished because of cell death (Figure 3A and 3B). At lower MOIs, there was a delay in time to peak of bioluminescence signal, likely because multiple CVB3-Luc replication cycles were required before the luciferase signal could be detected. For example, at MOI 5×10⁻⁴, ≈24 hours elapsed before onset of the bioluminescence signal. Treatment of hiPSC-CMs with decreasing MOI of CVB3-Luc also resulted in concentration-dependent reduction in pathological phenotype after 24 hours and a delay in cytopathic effect onset (Figure 3C). MOI 5×10⁻⁵ CVB3-Luc infection on 40,000 hiPSC-CMs still led to delayed well-wide cytopathic effect and complete hiPSC-CM death, suggesting that a single-digit number of viral particles is able to propagate the CVB3-Luc infection in a well of hiPSC-CMs (Figure 3D; Online Figure VIII). CVB3-Luc proliferation was substantially lower in HL-1 cells in comparison to hiPSC-CMs (Online Figure III). These results demonstrate that the hiPSC-CM/CVB3-Luc platform can be used to quantify viral replication in human cardiomyocytes.

Antiviral Drug Treatments Abrogate CVB3-Luc Proliferation
We used the hiPSC-CM/CVB3-Luc system to test the efficacy of antiviral compounds in abrogating viral proliferation in vitro (Table). We first treated hiPSC-CMs with IFNβ1, because IFNβ1 treatment is able to eliminate cardiotropic viruses in patients with myocarditis and reduce CVB3 proliferation in human embryonic stem cell–derived cardiomyocytes in vitro. We observed a concentration-dependent reduction in CVB3-Luc proliferation after 12-hour pretreatment with 0.5 to 8 ng/mL IFNβ1 (Figure 4A). At 8 ng/mL IFNβ1, we observed >50% reduction in CVB3-Luc proliferation on hiPSC-CMs (Figure 4B). However, IFNβ1 was unable to significantly...
reduce CVB3-Luc proliferation on HL-1 cells (Online Figure IX). When IFNβ1 was added concurrently with the virus, it was not as effective in reducing CVB3-Luc proliferation on hiPSC-CMs, perhaps because the 12-hour pretreatment allows time for the activation of IFNβ1 downstream pathways and expression of viral clearance proteins such as ribonuclease L and protein kinase R (Online Figure X).35 As a negative control, the antibiotic PenStrep did not cause a significant reduction in CVB3-Luc proliferation in hiPSC-CMs. Treatment with ≤8 ng/mL IFNβ1 alone did not cause significant alteration in hiPSC-CM cellular metabolism when measured with a WST-1 colorimetric assay (Figure 4C). IFNβ1 treatment at 8 ng/mL also did not induce visible alterations in hiPSC-CM monolayer morphology or beating patterns, suggesting that IFNβ1 reduces CVB3-Luc proliferation in hiPSC-CMs without inducing cardiotoxicity (Figure 4D).

Ribavirin is a nucleoside inhibitor that inhibits viral RNA synthesis.36 We observed a concentration-dependent reduction of CVB3-Luc proliferation on hiPSC-CMs infected with CVB3-Luc after 12-hour pretreatment with 25 to 800 μmol/L ribavirin (Figure 5A). Pretreatment with 800 μmol/L ribavirin caused ≈50% reduction in CVB3-Luc proliferation in hiPSC-CMs (Figure 5B). Similarly, ribavirin significantly reduced CVB3-Luc proliferation on HL-1 cells (Online Figure IX). Ribavirin was also moderately effective in reducing CVB3-Luc proliferation in hiPSC-CMs and HL-1 cells when added concurrently with CVB3-Luc, perhaps because as a small-molecule compound it is immediately able to function as a nucleoside inhibitor to reduce viral RNA transcription (Online Figure X). Ribavirin treatment ≤800 μmol/L did not cause significant alterations in hiPSC-CM metabolic output, as measured by WST-1 assay (Figure 5C), or any visible detrimental effects to hiPSC-CM morphology or beating patterns (Figure 5D).

Next, we tested the effect of fluoxetine, a selective serotonin reuptake inhibitor, in reducing CVB3-Luc replication. Fluoxetine is an effective inhibitor of coxsackievirus replication in HeLa cells.37 We treated hiPSC-CMs with fluoxetine at concentrations ranging from 1 to 32 μmol/L and observed that it had no significant effect in reducing CVB3-Luc proliferation at 1 to 2 μmol/L (Figure 6A and 6B). However, at 4 to 8 μmol/L fluoxetine (Figure 6B), pretreatment with 800 μmol/L ribavirin caused ≈50% reduction in CVB3-Luc proliferation in hiPSC-CMs (Figure 5B). Similarly, ribavirin significantly reduced CVB3-Luc proliferation on HL-1 cells (Online Figure IX). Ribavirin was also moderately effective in reducing CVB3-Luc proliferation in hiPSC-CMs and HL-1 cells when added concurrently with CVB3-Luc, perhaps because as a small-molecule compound it is immediately able to function as a nucleoside inhibitor to reduce viral RNA transcription (Online Figure X). Ribavirin treatment ≤800 μmol/L did not cause significant alterations in hiPSC-CM metabolic output, as measured by WST-1 assay (Figure 5C), or any visible detrimental effects to hiPSC-CM morphology or beating patterns (Figure 5D).

### Table. Antiviral Compounds Used to Abrogate CVB3-Luc Proliferation on hiPSC-CMs

<table>
<thead>
<tr>
<th>Antiviral Compound</th>
<th>Mechanism of Antiviral Action</th>
<th>Reduced CVB3-Luc Proliferation on hiPSC-CMs?</th>
<th>Cardiotoxicity Observed on hiPSC-CMs?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon β1</td>
<td>Activation of downstream viral RNA and protein clearance pathways</td>
<td>Yes</td>
<td>No</td>
<td>Scassa et al33; Kühl et al34</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>Nucleoside inhibitor of viral RNA synthesis</td>
<td>Yes</td>
<td>No</td>
<td>Heim et al36; Crotty et al47</td>
</tr>
<tr>
<td>Pyrrolidine dithiocarbamate</td>
<td>Inhibition of ubiquitin–proteasome proteolysis</td>
<td>Yes</td>
<td>No</td>
<td>Si et al38</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Mechanism unclear</td>
<td>Yes</td>
<td>No</td>
<td>Zuo et al37</td>
</tr>
</tbody>
</table>

CVB3-Luc indicates luciferase-expressing coxsackievirus B3 strain; and hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes.

---

Figure 4. Interferon β1 (IFNβ1) treatment reduces luciferase-expressing coxsackievirus B3 strain (CVB3-Luc) proliferation on infected human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) in a concentration-dependent fashion. A. Representative 96-well plate containing hiPSC-CMs infected with CVB3-Luc and pretreated with IFNβ1 for 12 h, visualized >12 h using bioluminescence imaging. B. Quantification of CVB3-Luc proliferation on hiPSC-CMs pretreated with IFNβ1. C. WST-1 assay quantifying cellular metabolic output and viability after treatment with increasing amounts of IFNβ1. D. Stills from videos of hiPSC-CMs treated with 8 ng/mL IFNβ1 for ≤72 h. These hiPSC-CMs continue beating after 72 h of IFNβ1 treatment.
fluoxetine, we observed a significant drop in CVB3-Luc proliferation with no cardiotoxicity observed at these concentrations (Figure 6B and 6C). At ≥16 μmol/L, fluoxetine was visibly toxic to hiPSC-CMs and resulted in decreased hiPSC-CM metabolic output and significant cell death (Figure 6C and 6D).

Finally, we tested the antiviral efficacy of the antioxidant PDTC, which reduces CVB3 replication through inhibition of the ubiquitin–proteasome proteolysis pathway. PDTC also inhibits the activation of 3Dpol, a viral RNA polymerase. We observed a concentration-dependent reduction in CVB3-Luc proliferation after 12-hour pretreatment of hiPSC-CMs with 25 to 800 μmol/L PDTC (Online Figure XI). Pretreatment with 800 μmol/L PDTC caused a significant reduction in CVB3-Luc proliferation (Online Figure XI).
PDTC significantly reduced CVB3-Luc proliferation on HL-1 cells (Online Figure IX). PDTC was also highly effective in reducing CVB3-Luc proliferation on hiPSC-CMs and HL-1 cells when added concurrently with CVB3-Luc or even after viral infection, reflective of its ability as a small-molecule compound to rapidly inhibit the activation of 3Dpol and stymie viral RNA transcription (Online Figure IX). When quantified using a WST-1 assay, higher concentrations of PDTC led to increased hiPSC-CM metabolic output (Online Figure XI). PDTC treatment for 72 hours at 800 μmol/L did not cause a visible detrimental alteration in hiPSC-CM morphology or beating patterns (Online Figure XI). Taken together, these results show that the hiPSC-CM/CVB3-Luc system can be used to screen for the functional efficacy and potential cardiotoxicities of antiviral compounds in vitro.

**hiPSC-CMs Provide Mechanistic Insight Into IFNβ1-Induced Viral Clearance Pathways**

We next assessed the specific molecular mechanisms by which hiPSC-CMs are able to clear viral mRNA and proteins to reduce coxsackievirus proliferation. We pretreated hiPSC-CMs with 8 ng/mL IFNβ1 for 12 hours and subsequently infected cells with CVB3-Luc (MOI 5) for 8 hours. Cells were then harvested for gene expression analysis using an Affymetrix DNA microarray. We observed that 139 genes were differentially expressed between infected hiPSC-CMs that did or did not receive IFNβ1 (Figure 7). Notably, IFNβ1 treatment significantly increased the expression of 22 genes previously implicated as mediators of viral clearance pathways (P<1.605E-31; Online Table II). These genes encode proteins that assist with a diverse range of antiviral functions such as ribonuclease activation, viral mRNA degradation, viral capsid protein degradation, viral particle sequestration, and interferon signal transduction. These results suggest that in hiPSC-CMs, IFNβ1 treatment activates a network of downstream antiviral genes that reduce CVB3-Luc proliferation after infection.

### Discussion

Viral myocarditis is a disease for which there remains no effective antiviral treatment. Our results suggest that hiPSC-CMs can be used to study the mechanisms of CVB3-induced viral myocarditis. Notably, hiPSC-CMs express CAR and are susceptible to coxsackievirus infection. We observed a down-regulation in CAR expression over a 15-day differentiation from hiPSCs to hiPSC-CMs, and previous studies have also illustrated that CAR mRNA expression decreases after 14 days of human embryonic stem cell to human embryonic stem cell–derived cardiomyocyte differentiation using embryoid body differentiation protocols. Adult cardiac tissues exhibit lower CAR mRNA levels compared with embryonic cardiac tissues, which is understandable given CAR’s established role for promoting proper embryonic cardiogenesis via maintaining myofibril integrity. However, we found that CAR expression is significantly lower in hiPSC-CMs in comparison with adult human left ventricular myocardium, perhaps reflecting the previously observed functional immaturity of hiPSC-CMs.

Cardiomyocytes can be infected by CVB3 and undergo apoptosis after completion of the CVB3 replication cycle. After CVB3 internalization in cardiomyocytes, viral protease 2A cleaves dystrophin, which connects the cardiomyocyte cytoskeleton with the extracellular matrix. This could impair the mechanical transduction of force normally generated by the sarcomeres. Cleavage of dystrophin also disrupts cell membrane integrity, allowing for efficient release of newly created CVB3 viral particles. Increased cell membrane permeability likely causes leakage of intracellular ions such as K⁺, Na⁺, and Ca²⁺ that maintain membrane potential and propagate the cardiac action potential necessary for normal cardiomyocyte excitation–contraction coupling. This interplay between viral protease–mediated disruption of intracellular structural proteins and subsequent loss of cell membrane integrity, combined with the viral load created during the
CVB3 replication cycle, likely results in the irregular beating, abnormal calcium transients, and eventual cessation of beating that we observed after CVB3 infection in hiPSC-CMs. Additionally, VP1, a component protein of the CVB3 capsid, shares ≤40% homology with cardiac myosin, and thus, antibodies against VP1 can cross-react with myosin.44 Indeed, we observed the VP1 stain intercalating with the cardiac tropinin T stain in the sarcomere. In spite of cross-reactivity, our VP1 antibody detects significant viral replication–induced increase in VP1 expression over time. We also observed a delayed cytopathic effect by infecting 40000 hiPSC-CMs with MOI 5×10⁻⁵ CVB3-Luc, suggesting that a single-digit number of viral particles (≈2 in a well of 40000 hiPSC-CMs) is able to propagate the viral infection across the entire well of hiPSC-CMs. This indicates that hiPSC-CMs are extremely susceptible to coxsackievirus infection.

There is a shortage of treatments for combating coxsackievirus infections and CVB3-induced myocarditis. Interferons have shown potential for eliminating cardiotropic viruses in vivo and in vitro and for improving ventricular function in patients with chronic myocarditis.10,33,34,36 Other compounds have shown modest effects in reducing CVB3 proliferation by interfering with viral capsid function.34 However, none of these therapies has received Food and Drug Administration approval, and thus, there is significant interest in finding safe and effective antiviral compounds for treating coxsackievirus infection and CVB3-induced myocarditis. The advantage of using hiPSC-CMs instead of commonly used cell lines such as HEK 293T or HeLa cells for antiviral drug screening is that hiPSC-CMs are more representative structurally and physiologically of the cardiac cell populations damaged during CVB3-induced myocarditis. Additionally, we think that hiPSC-CMs are superior to HL-1 cardiac cells for modeling CVB3 infection on cardiomyocytes and for antiviral drug screening. CXADR expression in hiPSC-CMs is substantially higher than in HL-1 cells and is also closer to CXADR levels found in primary human cardiac tissue. CVB3-Luc proliferates in hiPSC-CMs at a significantly higher level than in HL-1 cells. IFNβ1 was unable to reduce CVB3-Luc proliferation on HL-1 cells, suggesting that IFNβ1-mediated antiviral response pathways may be significantly altered in these cells. These discrepancies between hiPSC-CMs and HL-1s are likely because HL-1 cells are an immortalized mouse cardiac line derived from a tumor lineage and, therefore, may not be as useful as hiPSC-CMs for recapitulating adult human cardiomyocyte gene expression, viral infection, and drug responses. Testing novel therapeutics on hiPSC-CMs also allows for the assessment of potential cardiotoxicities and drug-induced arrhythmias, which are leading causes of drug withdrawal from the pharmaceutical market.45

We used CVB3-Luc to quantify the ability of a select group of compounds to reduce and delay coxsackievirus replication on hiPSC-CMs without inducing visible cardiotoxicity (Table). IFNβ1 was able to abrogate CVB3-Luc proliferation in a concentration-dependent manner and at very low concentrations, as demonstrated previously.35 Using a DNA microarray, we found that IFNβ1 reduces viral proliferation by priming hiPSC-CMs against viral infection and activating multiple genes associated with viral RNA and protein clearance (Figure 7; Online Table II).40 In infected hiPSC-CMs treated with IFNβ1, we detected a 10-fold upregulation in the expression of OAS1, which encodes for an activator of antiviral ribonuclease L.46 Additionally, we observed a 5-fold upregulation in the expression of EIF2AK2, which encodes for protein kinase R, an inhibitor of viral mRNA translation that acts by phosphorylating the eukaryotic transcription initiation factor (EIF2).40 Ribavirin and PDTC were also effective in inducing a concentration-dependent reduction of CVB3-Luc proliferation on hiPSC-CMs, because they are well-established inhibitors of viral replication.36,47 Importantly, IFNβ1, ribavirin, and PDTC did not induce significant cardiotoxic effects on hiPSC-CMs. Interestingly, PDTC caused an increase in hiPSC-CM metabolic output as measured by WST-1 assay, which we attribute to an off-target, PDTC-induced hyperactivation of mitochondrial dehydrogenase enzymes that are required to cleave WST-1 substrate into formazan dye. We observed significant hiPSC-CM death after treatment with high concentrations of fluoxetine, which in our assay reduced CVB3-Luc proliferation at 4 to 8 μmol/L. Fluoxetine overdose–induced arrhythmias have been reported previously but not cardiac cell death, as we have shown here.48 Although the aforementioned compounds were successful in reducing and delaying viral proliferation, we observed that a single-digit number of viral particles may be sufficient to propagate CVB3-Luc infection on an entire well of hiPSC-CMs, based on results from Figure 3D and Online Figure XIII. Thus, we confirmed the antiviral properties of 3 compounds and the potential cardiotoxicity of a fourth compound using the hiPSC-CM/CVB3-Luc system. We envision that this system could be used in a high-throughput manner to screen for novel, noncardiotoxic, antiviral therapeutics.

In summary, we have used hiPSC-CMs to model the mechanisms of viral myocarditis. Our results suggest that hiPSC-CMs express the CAR needed for CVB3 internalization and are susceptible to infection by CVB3. Gene networks known to be associated with viral mRNA and protein clearance are also activated after antiviral treatment in hiPSC-CMs infected with CVB3. Additionally, the hiPSC-CM/CVB3-Luc system can be used to screen for antiviral compound efficacy. Ultimately, we think that hiPSC-CMs represent a powerful tool for modeling disease mechanisms and for drug discovery purposes.49,50

Acknowledgments

We thank Frank van Kuppeveld (Department of Infectious Diseases and Immunology, University of Utrecht) for providing the CVB3-Luc plasmid from which the CVB3-Luc virus was produced. We thank Karim Majzoub for his assistance with viral primer design and proliferation assays. We thank Bhagat Patlolla for his help with obtaining the CVB3-Luc plasmid from which the CVB3-Luc virus was produced. We thank Bhagat Patlolla for his help with obtaining primary human left ventricular tissue samples. We thank the Stanford Neuroscience Microscopy Service (supported by NIH NS069375) for assistance with imaging.

Sources of Funding

We gratefully acknowledge funding support from the American Heart Association (AHA) Predoctoral Fellowship 13PRE15770000, National Science Foundation Graduate Research Fellowship Program DGE-114747 (A. Sharma), National Institutes of Health (NIH) Innovator Award DP2 OD004411, NIH U01 HL099776, CIRM RB3-05129 (S.M. Wu), and Leducq Foundation, AHA Established Investigator Award 14420025, NIH R01 HL113006, NIH U01 HL099776 (J.C. Wu).
Disclosures

None.

References


2. Dill GE Jr, Palacios BF, Falloon JD, Artzt HT, Mills J, Lee DC, Johnson RA. Active myocarditis in the spectrum of acute dilated cardiomyo-


18. Burridge PW, Keller G, Gold JD, Wu JC. Production of de novo cardiac myocytes: human pluripotent stem cell differentiation and direct repro-


**Novelty and Significance**

**What Is Known?**

- Viral myocarditis is a debilitating condition that can lead to cardiac arrhythmias and heart failure.
- Viral myocarditis can be caused by the B3 strain of coxsackievirus (CVB3), a common human pathogen that can rarely infect the heart and cause a life-threatening medical situation.
- It is difficult to obtain human cardiac tissues with which to study the mechanisms of cardiac viral infection, but thanks to recent discoveries in stem cell biology, we can now make an unlimited number of human heart cells (cardiomyocytes) produced from stem cells derived from a patient’s own skin or blood cells.

**What New Information Does This Article Contribute?**

- By acting as a model for true human heart tissue, human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can be used to study the mechanisms of viral myocarditis.
- These hiPSC-CMs express the proteins needed for CVB3 to enter the cardiomyocyte, and as a result, CVB3 can invade the cell and replicate rapidly within it.
- A genetically modified strain of CVB3 can be used in conjunction with hiPSC-CMs to quantify viral replication, and thus, this system can be used to test for the efficacy of antiviral compounds in reducing viral proliferation on human cardiomyocytes.

Viral myocarditis is a life-threatening cardiac disease that arises when the heart is infected by a virus such as CVB3. However, it is difficult to obtain human heart tissues with which to study the mechanisms of this disease because cardiac biopsies are invasive and expensive. Recent advances have allowed for the mass production of human heart cells from a patient’s own skin or blood samples. Using this hiPSC-CM technology, we were able to study the mechanisms of CVB3 infection on human cardiomyocytes. We found that hiPSC-CMs express the coxsackievirus receptor needed to internalize CVB3, and hiPSC-CMs are highly susceptible to CVB3 infection because the virus is able to proliferate rapidly and destroy the cells in a matter of hours. Notably, we used hiPSC-CM technology in conjunction with a genetically modified strain of CVB3 that expresses luciferase, a bioluminescent protein. This allowed us to quantify viral proliferation on hiPSC-CMs and screen for the antiviral efficacy of a panel of antiviral compounds. The CVB3/hiPSC-CM system that we established here could serve as a platform for discovering novel antiviral compounds that can effectively treat patients with viral myocarditis.
Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes as an In Vitro Model for Coxsackievirus B3–Induced Myocarditis and Antiviral Drug Screening Platform
Arun Sharma, Caleb Marceau, Ryoko Hamaguchi, Paul W. Burridge, Kuppusamy Rajarajan, Jared M. Churko, Haodi Wu, Karim I. Sallam, Elena Matsa, Anthony C. Sturzu, Yonglu Che, Antje Ebert, Sebastian Diecke, Ping Liang, Kristy Red-Horse, Jan E. Carette, Sean M. Wu and Joseph C. Wu

Circ Res. 2014;115:556-566; originally published online July 11, 2014;
doi: 10.1161/CIRCRESAHA.115.303810

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/6/556

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/07/11/CIRCRESAHA.115.303810.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

**Derivation of hiPSCs.** All the protocols for this study were approved by the Stanford University Institutional Review Board. Briefly, dermal fibroblasts were obtained via skin biopsy from 3 healthy patients in a previously described 7-patient family cohort. These samples were reprogrammed using a lentiviral vector expressing OKSM and were characterized as described previously. Three additional hiPSC lines were created from peripheral blood mononuclear cells using a Sendai virus vector expressing OKSM, as previously published. Subsequently, hiPSC colonies were picked and cultured on feeder-free, growth factor-reduced Matrigel-coated tissue culture dishes (BD Biosciences, San Jose, CA) with E8 pluripotent stem cell growth medium (STEMCELL Technologies, Vancouver, Canada).

**Immunofluorescence and laser confocal microscopy.** Beating hiPSC-CM sheets were incubated in TrypLE for 5 minutes followed by mechanical dissociation using a 200 μL pipettor and plated on 0.1-0.2% gelatin-coated glass coverslips. Immunostaining was performed according to previously established protocols. Primary antibodies consisted of mouse anti-human sarcomeric alpha-actinin (Sigma-Aldrich, St. Louis, MO), rabbit anti-human TNNT2 (Abcam, Cambridge, England), mouse anti-human TNNT (Abcam, Cambridge, England), rabbit anti-human alpha-smooth muscle actin (Abcam, Cambridge, England), mouse anti-enterovirus VP1 (Leica Microsystems, Buffalo Grove, IL), and rabbit anti-human CAR (Santa Cruz Biotechnology, Dallas, Texas). Goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594 (Invitrogen) were used as secondary antibodies. Imaging was performed using a DMIL -LED inverted tissue culture microscope (Leica Microsystems, Buffalo Grove, IL) or a Zeiss LSM 510Meta confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using Zen imaging software.
**Handling and processing of primary adult human left ventricle tissue samples.** All samples were obtained according to the guidelines of Stanford University IRB (Internal Review Board). Protocol #19810 “Collecting discarded tissue from organ donors and heart transplant recipients”. Left ventricle myocardial tissue samples were obtained from organ donors who have already been consented to use the excised tissue for scientific research purposes. Cardiac tissue was transported to the laboratory within 2 hours of procurement in ice-cold UW (University of Wisconsin) solution. Immediately, the specimen was transferred into a sterile container with UW solution for further processing. With clean forceps and scissors, the specimen was cut into < 500 mm³ cubes and placed into pre-labeled sterile 1.5 mL conical Eppendorf tubes and placed into a pre-chilled coolbox (CoolRack M15 by BioCision, LLC, CA) filled with liquid nitrogen for 12-15 minutes. Following this snap freezing step, the tubes were stored in a liquid nitrogen tank until needed for further processing. Upon thawing of the cardiac tissue sample from liquid nitrogen, the sample was homogenized first using a TissueRuptor system (Qiagen) and QIAshredder (Qiagen). RNA was then extracted with the miRNeasy kit (Qiagen) and utilized for downstream qRT-PCR as detailed previously.

**Culture of HL-1 Mouse Atrial Tumor Cardiac Cells.** HL-1 cells were cultured in Claycomb Medium (Sigma) + 10% FBS, 0.1 mM norepinephrine, and 2 mM L-glutamine as described previously⁴. Medium was changed daily. Cells were passaged using 0.25% Trypsin/EDTA prior to replating for experiments.
SUPPLEMENTAL FIGURE LEGENDS

Online Figure I: hiPSCs express intracellular sarcomeric proteins and CAR at cell-cell junctions. A, Table illustrating cell types and methods used to create hiPSCs from 6 healthy control individuals. Three hiPSC lines were created from human skin fibroblasts and were reprogrammed using the lentivirus method as previously described\(^1\). Three hiPSC lines were created from human peripheral blood mononuclear cells and reprogrammed using the Sendai virus method\(^2\). B, Representative hiPSC colonies from one of these healthy control individuals expresses typical markers for pluripotency, such as SSEA-4, Nanog, Tra-1-81, and Oct4 C, Healthy control hiPSCs also express CAR at cell-cell junctions.

Online Figure II: Characterization of hiPSCs and hiPSC-CMs from patients exhibiting DCM. A, hiPSCs were previously produced from individuals in a 7-member family cohort afflicted with familial dilated cardiomyopathy (DCM)\(^5\). These individuals exhibit a mutation in \(TNNT2\), encoding for a mutated version of cTnT (tryptophan in place of arginine at amino acid residue 173). hiPSCs from these individuals express typical markers for pluripotency, such as SSEA-4, Nanog, Tra-1-81, and Oct4. B, DCM hiPSCs express CAR at cell-cell junctions. C, DCM hiPSC-CMs exhibit abnormal sarcomeric organization, with “punctate” phenotypes for alpha-actinin and cTnT, as previously described\(^5\). D, DCM hiPSC-CMs, like healthy control hiPSC-CMs, express CAR at cell-cell junctions.

Online Figure III: HL-1 mouse atrial tumor cardiac cells express CAR but are less susceptible to CVB3-Luc infection than hiPSC-CMs. A, Immunofluorescence for CAR expression on HL-1 cells. Like hiPSC-CMs, HL-1 cells express CAR at cell-cell junctions. B,
Viral timecourse of HL-1 cells infected with MOI 5 CVB3-Luc. Immunofluorescence illustrates sparse VP1 staining in HL-1 cells, which has been previously described\(^6\). C, Representative 96-well plate containing hiPSC-CMs and HL-1 cells infected with CVB3-Luc at MOI 1. Each cell type was simultaneously plated at 40,000 cells and in quintuplicate. Cells were allowed to settle onto Matrigel-coated 96 wells for 24 hours before viral infection and bioluminescence imaging was conducted. D, Quantification of MOI 1 CVB3-Luc proliferation on hiPSC-CMs and HL-1 cells.

**Online Figure IV: Coxsackievirus and adenovirus receptor (CXADR) gene expression in hiPSC-CMs, HL-1 cells, and adult human left ventricle sample.** QRT-PCR results comparing expression of CXADR between day 15 hiPSC-CMs, HL-1 atrial tumor-derived cardiac cells, and primary adult human left ventricular (LV) myocardial tissue. Expression of CXADR in day 15 post-differentiation hiPSC-CMs is approximately 30-fold less than in LV tissue but is more than 10-fold greater than in HL-1 cells.

**Online Figure V: CVB3-Luc gene expression correlates with luciferase bioluminescent signal after hiPSC-CM infection.** QRT-PCR of CVB3-Luc gene expression after 0, 2, 4, 6, and 8 hours of MOI 5 infection on purified hiPSC-CMs. Primers were designed against coxsackievirus genes VP2, encoding for a viral capsid protein, and 3D, encoding for a viral RNA polymerase. Data points are normalized to the 8 hour-post infection timepoint. Luminescence readings were simultaneously taken at each time point and were also normalized to the 8 hour-post infection timepoint.
Online Figure VI: hiPSC-CMs from healthy control patients do not exhibit patient-specific differences in terms of CVB3-Luc viral response. Quantification for onset of cytopathic effect in 6 different sets of day 30 post-differentiation hiPSC-CMs infected with CVB3-Luc at MOI 5. Three lines were created from OKSM lentivirus reprogramming of human skin fibroblasts to hiPSCs. Three lines were created from OKSM Sendai virus reprogramming of human peripheral blood mononuclear cells to hiPSCs. All hiPSC lines were differentiated using a previously published, high efficiency, small molecule differentiation protocol.7

Online Figure VII: hiPSCs are susceptible to infection with CVB3-Luc. hiPSCs, like hiPSC-CMs, are susceptible to CVB3-Luc infection. Over 24 hours, there is an increase in VP1 expression. Cells continue to proliferate during the course of infection.

Online Figure VIII: Characterization of long-term, low MOI CVB3-Luc infection on hiPSC-CMs. Brightfield images of 40,000 hiPSC-CMs after 120 hours of infection with decreasing MOI of CVB3-Luc. All hiPSC-CMs in wells receiving MOI 5 to MOI 5x10^-5 CVB3-Luc succumbed to cytopathic effect by 120 hours post-infection. Asterisk represents the lowest MOI (5x10^-5) of CVB3-Luc on 40,000 hiPSC-CMs that is capable of inducing a cytopathic effect, suggesting that a single digit number of viral particles is able to propagate a CVB3-Luc infection to completely infect a well of hiPSC-CMs. Cytopathic effect is not observed at MOI 5x10^-6 and less.

Online Figure IX: Treatment of CVB3-infected HL-1 cells with antiviral compounds PDTC and Ribavirin, but not IFNβ1, reduces CVB3-Luc proliferation. A, Representative 96-well plate containing HL-1 cells infected with CVB3-Luc and treated with 8 ng/mL IFNβ1 at 12 hours
and 0 hours prior to MOI 1 CVB3-Luc infection visualized over 12 hours using bioluminescence imaging. **B**, Quantification of CVB3-Luc proliferation on HL-1 cells treated with 8 ng/mL IFNβ1. **C**, Representative 96-well plate containing HL-1 cells infected with CVB3-Luc and treated with 800 μM Ribavirin 12 hours and 0 hours prior to MOI 1 CVB3-Luc infection visualized over 12 hours using bioluminescence imaging. **D**, Quantification of CVB3-Luc proliferation on HL-1 cells treated with 800 μM Ribavirin. **E**, Representative 96-well plate containing HL-1 cells infected with CVB3-Luc and treated with 800 μM PDTC at 12 hours and 0 hours prior to MOI 1 CVB3-Luc infection visualized over 12 hours using bioluminescence imaging. **F**, Quantification of CVB3-Luc proliferation on HL-1 cells treated with 800 μM PDTC.

**Online Figure X**: Addition of antiviral compounds on hiPSC-CMs after CVB3-Luc infection is moderately effective in reducing CVB3-Luc proliferation. **A**, Representative 96-well plate containing hiPSC-CMs infected with CVB3-Luc and treated with 8 ng/mL IFNβ1 at 0, 2, 4, and 6 hours post-MOI 1 CVB3-Luc infection visualized over 12 hours using bioluminescence imaging. **B**, Quantification of CVB3-Luc proliferation on hiPSC-CMs treated with 8 ng/mL IFNβ1. **C**, Representative 96-well plate containing hiPSC-CMs infected with CVB3-Luc and treated with 800 μM Ribavirin at 0, 2, 4, and 6 hours post-MOI 1 CVB3-Luc infection visualized over 12 hours using bioluminescence imaging. **D**, Quantification of CVB3-Luc proliferation on hiPSC-CMs treated with 800 μM Ribavirin. **E**, Representative 96-well plate containing hiPSC-CMs infected with CVB3-Luc and treated with 800 μM PDTC at 0, 2, 4, and 6 hours post-MOI 1 CVB3-Luc infection visualized over 12 hours using bioluminescence imaging. **F**, Quantification of CVB3-Luc proliferation on hiPSC-CMs treated with 800 μM PDTC.
Online Figure XI: PDTC treatment reduces CVB3-Luc proliferation on infected hiPSC-CMs in a concentration-dependent fashion. A, Representative 96-well plate containing hiPSC-CMs infected with CVB3-Luc and pretreated with PDTC for 12 hours, visualized over 12 hours using bioluminescence imaging. B, Quantification of CVB3-Luc proliferation on hiPSC-CMs pretreated with PDTC. C, WST-1 assay quantifying cellular metabolic output and viability following treatment with increasing amounts of PDTC. D, Stills from videos of hiPSC-CMs treated with 800 μM PDTC for up to 72 hours. These hiPSC-CMs continue beating after 72 hours of PDTC treatment.
## SUPPLEMENTAL TABLES

**Online Table I: Primers Utilized for qRT-PCR.**

### TAQMAN PRIMERS

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Species</th>
<th>Gene Name</th>
<th>ABI Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXADR</td>
<td>Human</td>
<td>Coxsackievirus and Adenovirus Receptor</td>
<td>Hs00154661_m1</td>
</tr>
<tr>
<td>MYH6</td>
<td>Human</td>
<td>Myosin Heavy Chain 6, cardiac muscle, alpha</td>
<td>Hs00411908_m1</td>
</tr>
<tr>
<td>POU5F1</td>
<td>Human</td>
<td>POU class 5 homeobox 1</td>
<td>Hs00999632_g1</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Human</td>
<td>Troponin T Type 2 (Cardiac)</td>
<td>Hs00165960_m1</td>
</tr>
<tr>
<td>18S</td>
<td>Human</td>
<td>Eukaryotic 18S rRNA</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>CXADR</td>
<td>Mouse</td>
<td>Coxsackievirus and Adenovirus Receptor</td>
<td>Mm00438355_m1</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Mouse</td>
<td>Troponin T Type 2 (Cardiac)</td>
<td>Mm01290256_m1</td>
</tr>
<tr>
<td>18S</td>
<td>Mouse</td>
<td>Eukaryotic 18S rRNA</td>
<td>Mm03928990_g1</td>
</tr>
</tbody>
</table>

### SYBR GREEN PRIMERS

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Species</th>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2</td>
<td>Coxsackievirus</td>
<td>Forward</td>
<td>CGCTAGATTACTGCCCCTGGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTGGTGCCCTGCTAAACGTA</td>
</tr>
<tr>
<td>3D</td>
<td>Coxsackievirus</td>
<td>Forward</td>
<td>TGATCGCATCGTACCCATGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TAGGAAAGTGGCGTTGGTCC</td>
</tr>
<tr>
<td>18S</td>
<td>Human</td>
<td>Forward</td>
<td>AGAAACGGCTACCACATCCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CACCAGACTTGCCCTCCA</td>
</tr>
</tbody>
</table>

---

9
Online Table II: Antiviral defense genes upregulated after IFNβ1 treatment in infected hiPSC-CMs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Fold Change</th>
<th>ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISG15</td>
<td>ISG15 ubiquitin-like modifier</td>
<td>22.72</td>
<td>0.000804</td>
</tr>
<tr>
<td>IFIT3</td>
<td>interferon-induced protein with tetratricopeptide repeats 3</td>
<td>21.06</td>
<td>0.002703</td>
</tr>
<tr>
<td>IFIH1</td>
<td>interferon induced with helicase C domain 1</td>
<td>10.37</td>
<td>0.002208</td>
</tr>
<tr>
<td>OAS1</td>
<td>2'-5'-oligoadenylate synthetase 1, 40/46kDa</td>
<td>10</td>
<td>0.035424</td>
</tr>
<tr>
<td>IFITM1</td>
<td>interferon induced transmembrane protein 1</td>
<td>9.49</td>
<td>0.001991</td>
</tr>
<tr>
<td>DDX58</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 58</td>
<td>8.78</td>
<td>0.007341</td>
</tr>
<tr>
<td>IFI44L</td>
<td>interferon-induced protein 44-like</td>
<td>8.75</td>
<td>0.02334</td>
</tr>
<tr>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1, 91kDa</td>
<td>7.88</td>
<td>0.003083</td>
</tr>
<tr>
<td>OAS2</td>
<td>2'-5'-oligoadenylate synthetase 2, 69/71kDa</td>
<td>7.17</td>
<td>0.005251</td>
</tr>
<tr>
<td>ZC3HAV1</td>
<td>zinc finger CCCH-type, antiviral 1</td>
<td>7.07</td>
<td>0.00539</td>
</tr>
<tr>
<td>BST2</td>
<td>bone marrow stromal cell antigen 2</td>
<td>6.96</td>
<td>0.009572</td>
</tr>
<tr>
<td>IFIT1</td>
<td>interferon-induced protein with tetratricopeptide repeats 1</td>
<td>6.82</td>
<td>0.000477</td>
</tr>
<tr>
<td>MX1</td>
<td>myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)</td>
<td>6.77</td>
<td>0.008064</td>
</tr>
<tr>
<td>DDX60</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 60</td>
<td>5.59</td>
<td>0.003651</td>
</tr>
<tr>
<td>EIF2AK2</td>
<td>eukaryotic translation initiation factor 2-alpha kinase 2</td>
<td>5.47</td>
<td>0.004195</td>
</tr>
<tr>
<td>IRF9</td>
<td>interferon regulatory factor 9</td>
<td>5.24</td>
<td>0.000395</td>
</tr>
<tr>
<td>PLSCR1</td>
<td>phospholipid scramblase 1</td>
<td>4.15</td>
<td>0.000569</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>LogFC</td>
<td>p-value</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>TRIM25</td>
<td>tripartite motif containing 25, microRNA 3614</td>
<td>3.82</td>
<td>0.003324</td>
</tr>
<tr>
<td>HERC5</td>
<td>HECT and RLD domain containing E3 ubiquitin protein ligase 5</td>
<td>3.35</td>
<td>0.013563</td>
</tr>
<tr>
<td>IFIT5</td>
<td>interferon-induced protein with tetratricopeptide repeats 5</td>
<td>2.93</td>
<td>0.004233</td>
</tr>
<tr>
<td>SAMHD1</td>
<td>SAM domain and HD domain 1</td>
<td>2.82</td>
<td>0.027551</td>
</tr>
<tr>
<td>IFITM3</td>
<td>interferon induced transmembrane protein 3</td>
<td>2.74</td>
<td>0.002204</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL MOVIES

Online Movie I. Movie of beating sheet of hiPSC-CMs prior to purification via glucose starvation. These hiPSC-CMs spontaneously contract beginning at day 8-10 post-differentiation. Cell sheets consistently contain 85-95% hiPSC-CMs. Cells were recorded using a 10x objective.

Online Movie II. Movie showing a regularly beating monolayer of replated hiPSC-CMs following purification via glucose starvation. Cells were recorded using a 20x objective prior to MOI 5 CVB3-Luc infection.

Online Movie III. Movie showing an irregularly beating monolayer of replated hiPSC-CMs 6 hours following MOI 5 CVB3-Luc infection. Cells were recorded using a 20x objective.

Online Movie IV. Movie showing an irregularly beating monolayer of replated hiPSC-CMs 12 hours following MOI 5 CVB3-Luc infection. Cells were recorded using a 20x objective.
SUPPLEMENTAL REFERENCES


### A

<table>
<thead>
<tr>
<th>Cell Type Reprogrammed</th>
<th>Reprogramming Vector Used</th>
<th># Control hiPSC Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Skin Fibroblasts</td>
<td>Lentivirus</td>
<td>3</td>
</tr>
<tr>
<td>Peripheral Blood Mononuclear Cells</td>
<td>Sendai virus</td>
<td>3</td>
</tr>
</tbody>
</table>

### B

#### SSEA-4/Nanog
- Phase
- SSEA-4
- Nanog
- DAPI

#### TRA-1-81/Oct4
- Phase
- TRA-1-81
- Oct4
- DAPI

### C

#### Coxsackievirus and Adenovirus Receptor
- Phase
- CAR
- DAPI
Relative Expression

**CXADR**

- hiPSC-CM d15
- HL-1
- LV

Online Figure IV
Normalized Luciferase Luminescence

Normalized Viral Gene Expression

VP2 Primers ($R^2=0.99$)

3D Primers ($R^2=0.99$)

0 Hrs Post-Infection

2 Hrs Post-Infection

4 Hrs Post-Infection

6 Hrs Post-Infection

8 Hrs Post-Infection

Online Figure V
Online Figure VI

Time Until Cytopathic Effect Observed (Hours after CVB3-Luc MOI 5 Infection)

- hiPSC-CM Lentivirus Line 1
- hiPSC-CM Lentivirus Line 2
- hiPSC-CM Lentivirus Line 3
- hiPSC-CM Sendai virus Line 1
- hiPSC-CM Sendai virus Line 2
- hiPSC-CM Sendai virus Line 3
Online Figure VII
CVB3-Luc on 4x10^4 hiPSC-CMs (120 hours post-infection)

Online Figure VIII
IFNβ1 on HL-1 cells
Ribavirin on HL-1 cells
PDTC on HL-1 cells

Online Figure IX
Online Figure X