Studying arrhythmogenic right ventricular dysplasia with patient-specific iPSCs

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Cellular reprogramming of somatic cells to patient-specific induced pluripotent stem cells (iPSCs) enables in vitro modelling of human genetic disorders for pathogenic investigations and therapeutic screens1-7. However, using iPSC-derived cardiomyocytes (iPSC-CMs) to model an adult-onset heart disease remains challenging owing to the uncertainty regarding the ability of relatively immature iPSC-CMs to fully recapitulate adult disease phenotypes. Arrhythmogenic right ventricular dysplasia/cardio myopathy (ARVD/C) is an inherited heart disease characterized by pathological fatty infiltration and cardiomyocyte loss predominantly in the right ventricle2, which is associated with life-threatening ventricular arrhythmias. Over 50% of affected individuals have desmosome gene mutations, most commonly in PKP2, encoding plakophilin-2 (ref. 9). The median age at presentation of ARVD/C is 26 years2. We used previously published methods8,9 to generate iPSC lines from fibroblasts of two patients with ARVD/C and PKP2 mutations10,11. Mutant PKP2 iPSC-CMs demonstrate abnormal plakoglobin nuclear translocation and decreased β-catenin activity12 in card iogenic conditions; yet, these abnormal features are insufficient to reproduce the pathological phenotypes of ARVD/C in standard cardiogenic conditions. Here we show that induction of adult-like metabolic energetics from an embryonic/glyc olytic state and abnormal peroxisome proliferator-activated receptor gamma (PPAR-γ) activation underlie the pathogenesis of ARVD/C. By co-activating normal PPAR-alpha-dependent metabolism and abnormal PPAR-γ pathway in beating embryoid bodies (EBs) with defined media, we established an efficient ARVD/C in vitro model within 2 months. This model manifests exaggerated lipogenesis and apoptosis in mutant PKP2 iPSC-CMs. iPSC-CMs with a homozygous PKP2 mutation also had calcium-handling deficits. Our study is the first to demonstrate that induction of adult-like metabolism has a critical role in establishing an adult-onset disease model using patient-specific iPSCs. Using this model, we revealed crucial pathogenic insights that metabolic derangement in adult-like metabolic milieu underlies ARVD/C pathologies, enabling us to propose novel disease-modifying therapeutic strategies.

Mutations resulting in ARVD/C are frequently found in five components of the cardiac desmosome, which include junction plakoglobin, plakophilin-2 (PKP2), desmoplakin (DSP), desmoglein-2 and desmocollin-2. Pathological hallmarks of ARVD/C are progressive fibro-fatty replacement of cardiomyocytes with increased cardiomyocyte apoptosis primarily in the right ventricle. Pathogenic processes of ARVD/C are difficult to study because obtaining cardiac samples from early stages of human ARVD/C hearts is not possible owing to ARVD/C being commonly diagnosed at advance diseased stages or post-mortem2, and because primary cardiac tissues are difficult to biopsy safely from symptomatic ARVD/C patients owing to the risk of cardiac perforation. Moreover, mouse models of ARVD/C remain unproven for finding clinically feasible therapy. These limiting factors impose significant constraints in developing therapies for human ARVD/C.

Using four retroviral vectors containing Oct4, Sox2, Klf4 and cMyc, we first generated several iPSC lines from fibroblasts of a patient with clinical ARVD/C and a homozygous c.2484C>T mutation in PKP2 that causes cryptic splicing with a 7-nucleotide deletion in exon 12, leading to frame-shift of the carboxy-terminal amino acids11. Three iPSC lines were extensively characterized: JK#2, JK#7 and JK#11 (Fig. 1 and Supplementary Figs 1 and 2). All iPSC clones expressed pluripotent markers, including Oct4, Tra1-81, Nanog and SSEA4, had normal karyotypes, and formed teratoma when injected into severe combined immunodeficient (SCID) mice. Pluripotent status of mutant PKP2-iPSCs was also demonstrated by the methylation patterns of Nanog promoter regions (Fig. 1c) in comparison to parental mutant PKP2 fibroblasts and H9 human embryonic stem cells (hESCs). Global transcriptome expression profiles showed that all mutant PKP2-iPSC lines showed a higher degree of similarity with H9 hESCs and normal iPSCs (CF-iPSCs, female, characterized in Supplementary Fig. 3) than their parent fibroblasts (Fig. 1f). Importantly, all three JK mutant iPSC lines showed silenced exogenous retroviral transgenes and activation of endogenous pluripotent genes (Supplementary Fig. 2). Genomic DNA sequencing and messenger RNA analysis confirmed the homozygous c.2484C>T mutation in PKP2 that caused predominantly cryptic splicing in exon 12 of three JK lines (Supplementary Figs 1d and 2b). These results demonstrated the successful reprogramming of parental mutant PKP2 fibroblasts to iPSC lines.

We differentiated human H9 hESCs, CF-iPSCs and mutant PKP2-iPSCs to beating EBs (Supplementary Videos 1 and 2) using a standard cardiogenic protocol12. Consistent with previous findings in ARVD/C cell line and mouse models with desmoplakin downregulation/deletion13,14, we observed abnormal nuclear translocation of junction plakoglobin proteins (PKG) (Fig. 2a, b), and very low β-catenin activity and expression in mutant iPSC-CMs (Supplementary Fig. 4) but not in normal hESC-CMs under baseline cardiogenic conditions. These results indicate that mutant PKP2 proteins with frame-shifted C-terminals fail to anchor PKG to the sarcolemmal membrane, resulting in PKG nuclear translocation and downregulation of β-catenin activities15. However, no exagerrated lipogenesis (by Nile Red staining) or apoptosis (by TdT-mediated dUTP nick end labelling (TUNEL) assay) in H9 hESC- or mutant iPSC-CMs was found after culturing for 2–3 months in this baseline cardiogenic condition (Fig. 2c), an observation that is consistent with the delayed, adult-onset clinical course of ARVD/C.

We then focused our studies on the cardiomyocyte portions (positive for cardiac α-actinin or troponin I (CTNl)) of beating EBs. We counted (1) the percentage of cardiomyocytes that possessed positive TUNEL nuclear staining as the cardiomyocyte (CM) apoptotic index, and (2) the percentage of cardiomyocytes that contained any Nile-Red-positive

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lipid droplets as the indicator of lipogenesis in cardiomyocytes. The baseline apoptotic index and percentages of lipid-laden cardiomyocytes are 1.8 ± 0.3 and 0.8 ± 0.5% for 60-day-old hESC-CMs, and 2.1 ± 0.6 and 0.9 ± 0.1% for 60-day-old mutant iPSC iPSC-CMs (no statistical difference between these two groups), respectively. Because the major metabolic differences between embryonic and adult cardiomyocytes are (1) embryonic cardiomyocytes use mostly glycolysis for energy production, and (2) adult cardiomyocytes produce most energy via fatty acid oxidation (FAO) but retain capacity to readily switch to glucose or other substrate utilization when fatty acid is not available or FAO is compromised. We first created a lipogenic milieu in beating EBs with an adipogenic cocktail (insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX), termed the 3-factor (3F) protocol) in an attempt to induce adult-like energy metabolism and accelerate pathogenesis in mutant iPSC-CMs. With this 3F protocol, we observed mildly increased lipogenesis with minimal apoptosis after 4–5 weeks of treatment (13.2 ± 4.3 and 4.8 ± 1.0% lipid-laden cardiomyocytes and 6.3 ± 1.4 and 3.9 ± 1.8% apoptotic cardiomyocytes in hESC-CMs and mutant PKP2 iPSC-CMs, respectively, P = non-significant between groups, Fig. 2d–k). Notably, this 3F protocol induced more expression of PPAR-alpha (PPAR-α), the major transcriptional regulator of fatty acid metabolism in adult cardiomyocytes, in mutant iPSC-CMs than in hESC-CMs (Fig. 2g). PPAR-γ, which should be minimally activated in normal cardiomyocytes, was slightly activated by this 3F protocol in both beating EBs. Because PPAR-γ pathway has been reported to be abnormally hyperactivated in right ventricle tissue samples of ARVD/C hearts, we first created a lipogenic milieu in beating EBs, and (2) adult cardiomyocytes produce most energy via fatty acid metabolism, but rosiglitazone and indomethacin are not natural human ligands. Therefore, we searched for a natural ligand that could replace rosiglitazone and indomethacin for inducing abnormal PPAR-γ activation. We found that the endogenous PPAR-γ activator 13-hydroxyocta-decadienoic acid (13-HODE), a major component of the oxidized low-density lipoprotein (oxLDL), could replace rosiglitazone and indomethacin in the 5F protocol for abnormal PPAR-γ
Figure 2 | Induction of pathognomonic features of ARVD/C using mutant PKP2-iPSCs. a, b, H9 hESC-CMs showed sarcolemmal connexion 43 (CX43) and PKG staining (red) at both cell membrane and nucleus (DAPI) (a); yet PKG staining in mutant iPSC-CMs is restricted to the nuclei (b). c, At baseline, minimal TUNEL, nuclear staining (apoptosis) or Nile Red labelling (lipogenesis) was found in mutant iPSC-CMs. Pictures with low magnification are used to show the lack of pathologies. d, Protocols for lipogenic (3F) and pathogenic (5F) induction. e, f, Minimal apoptosis (e) and mild increase of lipid-laden (red) CMs (f) were found after 3F treatment. g, Summary of mRNA expression levels of PPAR-α and PPAR-γ (relative to GAPDH) in beating EBs. PPAR-α: 4.3 ± 0.3 × 10⁻³ and PPAR-γ by 5F demonstrated overall depressed energy metabolism with 86% reduction in FAO and 74% reduction in glycolysis, resulting in a fuel shift from using both fatty acids and glucose to dominant glucose utilization (including glycolysis and pyruvate oxidation), much like the so-called metabolic burnt-out state observed in failing hearts^22 (Fig. 4). This fuel-shift after pathogenic induction is accompanied by significant downregulation of mRNA transcripts of carnitine palmitoyltransferase-1B (CPT1B) and pyruvate dehydrogenase kinase-4 (PDK4), leading to inhibition of FAO and activation of pyruvate dehydrogenase (PDH) for increased pyruvate oxidation^16,26, respectively (Fig. 4i–k). Thus, results from immunocytochemical, genetic and metabolic assays support strongly that coactivation of PPAR-α and PPAR-γ by hormones and small molecules accelerates the pathogenesis and establishes an efficient in vitro model of ARVD/C, recapitulating the metabolic and pathological signatures of failing ARVD/C hearts within 2 months.

We also used an episomal method^10 to generate three lines of genome-integration free iPSC (SW#5, SW#6 and SW#7) from a second patient with clinical ARVD/C (Supplementary Fig. 9) and a heterozygous c.2013delC in exon 10 of PKP2 (termed delC PKP2 mutation) that causes frame-shift and premature termination in exon 10. Confirmation of pluripotent status and the heterozygous c.2013delC in PKP2 of these three SW iPSC clones are shown in Supplementary Figs 10 and 11. Prematurely-terminated delC mutant PKP2 transcripts are probably unstable and degraded to undetectable levels (see Supplementary Figs 10b, c and 11h). SW iPSC-CMs also showed increased nuclear PKG translocation (Supplementary Fig. 12a–c) and very low β-catenin nuclear localization (Supplementary Fig. 4) as described for the homozygous c.2484C>T mutant PKP2 iPSC-CMs. We induced ARVD phenotypes from SW#5 and SW#7 mutant iPSC-CMs but not in normal female activation, leading to extensive lipogenesis (37.5 ± 6.0%) and apoptosis (22.4 ± 4.0%) in mutant iPSC-CMs (Fig. 3e). Moreover, to support the crucial pathogenic role of coactivation of abnormal PPAR-α and normal PPAR-γ pathways in ARVD/C, we showed that blockade of PPAR-γ overactivation with PPAR-γ antagonists, GW9662 (GW) or T0070907 (T007)23, during pathogenic induction with media containing 13-HODE and 3-factors, prevents apoptosis and lipogenesis in mutant iPSC-CMs (Fig. 3f–h). Also, blockade of PPAR-α activation with an antagonist, 2 μM GW6471, after 5F treatment (Supplementary Fig. 6) or activation of PPAR-γ alone (Supplementary Fig. 5e–g) did not induce significant ARVD/C pathologies, supporting the crucial role of PPAR-α/PPAR-γ coactivation in ARVD/C pathogenesis. Significant FAO and β-oxidation activated by PPAR-α seem to be required for cardiomyocyte apoptosis as well (Supplementary Fig. 6b–e). Furthermore, because mitochondrial function is tightly linked to energy metabolism and apoptosis^24, we decreased reactive oxygen species (ROS) production from mitochondria with ROS scavengers, 1 mM N-acetyl-cysteine or ascorbic acid individually, in the 5F pathogenic assays of FAO and glycolysis in live cells showed that mutant PKP2 iPSC-CMs and normal iPSC-CMs derived from a second normal iPSC line (hS-iPSC, male, characterized in Supplementary Fig. 7) had dominant glycolytic energetics (an embryonic pattern^25) at the baseline (Fig. 4 and Supplementary Fig. 8). After activation of PPAR-α by 3F, mutant and normal iPSC-CMs had similar levels of glycolysis but significant activation of FAO (an adult-like pattern) when compared to the non-induced, baseline conditions (zero factor). Compared to 3F induction, mutant PKP2 iPSC-CMs after pathogenic coactivation of H9 hESC-CMs showed sarcolemmal connexion 43 (CX43) and PKG staining (red) at both cell membrane and nucleus (DAPI) (a); yet PKG staining in mutant iPSC-CMs is restricted to the nuclei (b). At baseline, minimal TUNEL, nuclear staining (apoptosis) or Nile Red labelling (lipogenesis) was found in mutant iPSC-CMs. Pictures with low magnification are used to show the lack of pathologies. Protocols for lipogenic (3F) and pathogenic (5F) induction. Minimal apoptosis (e) and mild increase of lipid-laden (red) CMs (f) were found after 3F treatment. Summary of mRNA expression levels of PPAR-α and PPAR-γ (relative to GAPDH) in beating EBs. PPAR-α: 4.3 ± 0.3 × 10⁻³ and PPAR-γ by 5F demonstrated overall depressed energy metabolism with 86% reduction in FAO and 74% reduction in glycolysis, resulting in a fuel shift from using both fatty acids and glucose to dominant glucose utilization (including glycolysis and pyruvate oxidation), much like the so-called metabolic burnt-out state observed in failing hearts (Fig. 4). This fuel-shift after pathogenic induction is accompanied by significant downregulation of mRNA transcripts of carnitine palmitoyltransferase-1B (CPT1B) and pyruvate dehydrogenase kinase-4 (PDK4), leading to inhibition of FAO and activation of pyruvate dehydrogenase (PDH) for increased pyruvate oxidation, respectively (Fig. 4i–k). Thus, results from immunocytochemical, genetic and metabolic assays support strongly that coactivation of PPAR-α and PPAR-γ by hormones and small molecules accelerates the pathogenesis and establishes an efficient in vitro model of ARVD/C, recapitulating the metabolic and pathological signatures of failing ARVD/C hearts within 2 months.

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**Figure 3** Rescue of pathological features of mutant PKP2-iPSC-CMs.

- **a)** Diagram of lentiviral vectors used to stably integrate control-GFP or WT-PKP2-GFP into mutant iPSC-CMs. **b)** Top panels, PKG (red) remained restricted to nuclei with control-GFP vectors; bottom panels, PKG distributed only in high extracellular Ca²⁺, but not in WT

- **c)** After 5F and WT PKP2-GFP lentiviral infection, TUNEL staining could only be found in WT-PKP2-GFP-positive CMs (left, 30.3 ± 41.0) compared to H9 hESC-CMs in baseline conditions, mutant iPSC-CMs demonstrated mildly decreased mRNA expression levels of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA, for Ca²⁺ re-uptake) with preserved Na⁺/Ca²⁺ exchanger 1 (NCX1, for Ca²⁺ extrusion) expression. **d)** After 5F pathogenic induction, mutant iPSC-CMs demonstrated a prolonged [Ca²⁺]i relaxation phase evan in normal Ca²⁺ media after pacing (Supplementary Fig. 13a–d), indicating normal [Ca²⁺]i handling capability. Furthermore, using puromycin-purified cardiomyocytes and compared to hESC-CMs, qRT–PCR analysis of mutant iPSC-CMs in baseline conditions showed mildly decreased mRNA expression levels of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA, for Ca²⁺ re-uptake) with preserved Na⁺/Ca²⁺ exchanger 1 (NCX1, for Ca²⁺ extrusion) expression, which may explain the impaired [Ca²⁺]i relaxation of mutant iPSC-CMs even in normal extracellular Ca²⁺ media at baseline. In contrast, after 5F treatment, both NCX1 and SERCA expression levels were significantly downregulated (Supplementary Fig. 13e, f) relative to hESC-CMs, which would account for the further impaired [Ca²⁺]i relaxation phase of mutant iPSC-CMs even in normal Ca²⁺ media. Also, electrophysiological

CF-iPSC-CMs with the 5F protocol. Only the 5F protocol induced coactivation of normal PPAR-γ and abnormal PPAR-α pathways that led to exaggerated lipidogenesis and cardiomyocyte apoptosis in delC PKP2-iPSC-CMs (Supplementary Fig. 12), consistent with the results shown in Fig. 2. The second hS-iPSC line was also used to show that the 5F protocol did not induce exaggerated lipidogenesis or cardiomyocyte apoptosis in normal male iPSC-CMs (Supplementary Fig. 7f).

We further used JK mutant iPSC-CMs to investigate other cardiomyocyte properties that may contribute to pathological features. Compared to H9 hESC-CMs in baseline conditions, mutant iPSC-CMs demonstrated slower intracellular calcium ([Ca²⁺]i) relaxation after pacing only in high extracellular Ca²⁺ media (5.8 mM) with a prolonged relaxation time constant, but not in normal Ca²⁺ media (1.8 mM). However, after 5F pathogenic induction, mutant iPSC-CMs demonstrated a prolonged [Ca²⁺]i relaxation phase even in normal Ca²⁺ media after pacing (Supplementary Fig. 13a–d), indicating normal [Ca²⁺]i handling capability. Furthermore, using puromycin-purified cardiomyocytes and compared to hESC-CMs, qRT–PCR analysis of mutant iPSC-CMs in baseline conditions showed mildly decreased mRNA expression levels of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA, for Ca²⁺ re-uptake) with preserved Na⁺/Ca²⁺ exchanger 1 (NCX1, for Ca²⁺ extrusion) expression, which may explain the impaired [Ca²⁺]i relaxation of mutant iPSC-CMs even in normal extracellular Ca²⁺ media at baseline. In contrast, after 5F treatment, both NCX1 and SERCA expression levels were significantly downregulated (Supplementary Fig. 13e, f) relative to hESC-CMs, which would account for the further impaired [Ca²⁺]i relaxation phase of mutant iPSC-CMs even in normal Ca²⁺ media. Also, electrophysiological
properties of JK PKP2 mutant iPSC-CMs are different from H9 hESC-CMs (Supplementary Fig. 14) at baseline and show further impairment after PPAR-α and PPAR-γ coactivation. Future mechanistic studies need to determine the roles of impaired [Ca$^{2+}$]i handling and altered electrical properties in mediating pathologies or arrhythmia of mutant iPSC-CMs.2-27.

Finally, to explain the predominant pathology in the right ventricle of ARVD/C hearts, we increased the number of Isl1-positive cardiac progenitor cells and, subsequently, right-ventricle-like cardiomyocytes. After 5F pathogenic induction, EBs with enriched Isl1+ cells had significantly more lipogenesis and apoptosis in cardiomyocytes than EBs with minimal numbers of Isl1+ progenitor cells (Supplementary Fig. 15d–g). This result supports the notion that Isl1+ cells confer the dominant pathologies in the right ventricle.20.

In conclusion, using patient-specific mutant PKP2-iPSCs and various pathogenic conditions, we accelerate the pathogenesis of an adult-onset disease. We demonstrate the importance of PPAR-α/PPAR-γ coactivation, ROS production, fatty acid oxidation and Isl1+ cells in the pathogenesis of ARVD/C. These efficient in vitro iPS models recapitulate the pathognomonic features of ARVD/C and enable pathogenic investigation and therapeutic screens.

**METHODS SUMMARY**

The retroviral and episomal methods of generating iPSCs from normal and patients’ fibroblasts were performed as published previously.2,3 Human ESC and iPSC culture methods, lentiviral transduction and derivation of cardiomyocytes using standard cardiogenic protocols were described previously.4 ARVD/C fibroblasts were obtained from Johns Hopkins University and normal fibroblasts from UCSD with approved protocols at each institution. Genetic sequencing and
Karyotyping was performed by Cell Line Genetics. Standard techniques were used with the StatView/JMP program (SAS Institute) and presented as mean ± s.e.m. \( [\text{Ca}^{2+}]_i \) relaxation curve fitting was performed with SigmaPlot 8.0 software using the Marquardt-Levenberg algorithm to achieve the best fit. Statistical difference was analysed by an ANOVA procedure for multiple comparisons and by the unpaired \( t \)-test for pair-wise comparisons with a \( P \)-value \( < 0.05 \) considered statistically significant.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions C.K. and H.-S.V.C. designed experiments and wrote the manuscript; J.E.M., H.C. and D.P.J. provided clinical assessment and patient’s fibroblasts as well as analysis of data and assistance with preparation of the manuscript; C.K., J.Wo., S.W., C.W., S.S., N.G.K. and H.-S.V.C. performed the experiments; S.F. and P.L.P. helped C.K. in performing bisulphide sequencing; J.Wo. analysed microarray, Seahorse and immunocytochemical data; D.P.K. and T.C.L. provided scientific advice and primers for metabolic assays. This work was supported by NIH grants (RO1 HL058493 and RO1 HL101189) (to D.P.K.); NIH grants (RO1 AR056712 and RO1 AR052779) (to P.L.P); California Institute of Regenerative Medicine (CIRM) grants (RS1-00171-1, RB2-01512 and RB4-06278) and NIH grant (RO1 HL05194) to H.-S.V.C.

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METHODS

Culture conditions and generation of PKP2 iPSCs. We obtained normal and mutant PKP2 human fibroblasts from University of California-San Diego and Johns Hopkins University after informed consent was obtained from all subjects under approved study protocols by respective Institutional Review Boards at both universities and SBMRI. The clinical presentation, diagnostic data and genetic analysis of the first patient and her family members were published previously11. Normal fibroblasts were obtained from discarded heart and skin of aborted fetuses of pregnant females without ARVD/C and any family history of ARVD/C. HEK 293T cells and human fibroblasts were cultured in Dulbecco’s modified Eagle media (DMEM, HyClone) containing 10% heat-inactivated fetal bovine serum (FBS), 1 mM non-essential amino acids (NEAAs), 1× GlutaMAX, and 100 unit ml⁻¹ penicillin with 100 μg ml⁻¹ streptomycin (penicillin/streptomycin). Human iPSCs and h9F hESC were cultured on irradiated mouse embryonic fibroblasts (MEF) feeders in stem cell media containing Knockout (KO)-DMEM, 20% KO Serum Replacement (SR), 1 mM NEAAs, 1× GlutaMAX, 0.1 mM β-mercaptoethanol, penicillin/streptomycin, and 8 ng ml⁻¹ bFGF (Sigma). Retroviral plasmids, pMXs-hc-MYC, pMXs-hSOX2, pMXs-hKLF4, and pMXs-hOCT3/4, were purchased from Addgene. The protocol to generate iPS cells with retroviral plasmids is essentially the same as previously published1. Briefly, each pMXs retroviral vector along with pMig gag-pol and PCMV-VSV-G plasmids at the ratio of 2:2:1 (total 20 μg) were cotransfected into HEK-293T cells for viral particle production. At 36 h after transfection, DMEM containing viruses from each plate were collected, mixed with 8 μg ml⁻¹ polybrene (Sigma), and immediately used to infect human fibroblasts (100,000 cells) by spinning transduction. Six days after the first infection, a half-million infected fibroblasts were trypsinized and plated on 100-mm dishes containing irradiated MEF feeder layers in 10% FBS-containing DMEM. One day later, the media was changed to stem cell media as described above. Induced pluripotent colonies were selected based on stem cell-like morphologies at 12–14 days after the initial infection. All culture media and reagents were purchased from Invitrogen unless indicated otherwise.

The episomal method of generating integration-free iPSC colonies is the same as published previously by using pCXLE-EGFP, pCXLE-hOCT3/4, pCXLE-hSK and pCXLE-hUL (Addgene) episomal vectors10.

Cardiomyocyte differentiation. Our standard cardiogenic protocol to produce beating EBs was published previously11, 12 and the media was EB media: KO-DMEM with 20% FBS, 1 mM NEAAs, 1× GlutaMAX, 1 mM β-mercaptoethanol and penicillin/streptomycin. A modified method published previously13 to enrich cardiac progenitor cells was also used and optimized for our hESCs and iPSCs. After beating EBs were observed, they were maintained in the EB media containing only 2% FBS. The method to purify cardiomyocyte spheroids (CSs) from EBs containing α-MHC-Puro⁺ was published previously14,15. Adipogenic conditions and pharmacological investigation. In the presence of very low serum concentrations (2% FBS), we used low micromolar concentrations of insulin (50 μg ml⁻¹) and dexamethasone (0.5 μM) as well as 0.25 mM IBMX (3F protocol) to induce adipogenic environment in beating EBs. 5 μM rosiglitazone (Cayman Chemical) and 200 μM indomethacin were added to the 3F protocol to form the 5F protocol. Beating EBs at 30 days of differentiation were treated with 3F- or 5F-media for 4–5 weeks before further investigation. 20 μg ml⁻¹ 13-HODE (Cayman) was used to activate PPAR-γ based on published dose–response data13. 3 μM GW9662 (Cayman) or 0.5 μM T0070907 (Cayman)15 was chosen to block 13-HODE action based on their half maximal inhibitory concentrations of specific inhibition of PPAR-γ activation and assumed competitive antagonism from 13-HODE. N-acetyl-cysteine16, ascorbate (1 mM)17, BIO (Tocris, 1-2.5 μM)17 and Dickkopf-1 (Invitrogen)20 were used according to previous publications. All pharmacological agents were purchased from Sigma unless otherwise indicated.

Karyotype analysis and teratoma formation. Karyotype analysis was performed by Cell Line Genetics. For teratoma formation, we injected −10 unit undifferentiated human iPSCs under the kidney capsule of SCID beige mice (Harlan Laboratories). Tumors were collected 8 weeks after injection for histological sections as well as haematoxylin and eosin staining by the SBMRI Histology Core facility. All animal protocols were approved by the SBMRI Animal Care and Use Committee. DNA microarray analysis. Total DNA from JK#2, JK#11 and SW#5, SW#6 and SW#7 clones was prepared using mirVana miRNA Isolation Kit (Ambion) from 5–10 EBs or puromycin-selected CSs after respective treatments. Complementary DNA (cDNA) was synthesized by the Quant iTect RT kit (Qiagen). Quantitative real-time polymerase chain reaction (qRT–PCR) was performed on a Roche LightCycler 480 using the LightCycler 480 SYBR Green I Master kit (Roche) according to protocols recommended by the manufacturer. The cycling conditions were 40 cycles (95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s). Quantification was carried out by correcting for amplification efficiency of the primer using a standard curve, followed by normalizing transcript levels to the amount of total ubiquitously expressed GAPDH transcripts. Two physical replicates from each of ≥3 respective sets of experiments were used to construct the histograms in all figures. All qRT–PCR and regular reverse transcription PCR (RT–PCR) primers are listed in Supplementary Tables 1 and 2.

Genomic and cDNA sequence analysis. Genomic DNA from three to five colonies of undifferentiated H9 and PKP2-iPSCs was collected using QIAamp DSP DNA Mini Kit (Qiagen). Genomic DNA of the PKP2 gene region was amplified with 5'-TGATGAAATAGGTTCGGATGT (sense) and 5'-CTGAGGG GTACCGACATGTCTG (antisense)18 by PCR (95 °C for 2–3 min; 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; followed by 5 min at 72 °C). PCR products were directly used for sequencing with two primers as published previously11.

For the expressed PKP2, cDNA was synthesized with Oligo-dT primer and Superscript III reverse transcriptase (Invitrogen) from puromycin-purified hESC- or PKP2-iPSC-Cs. The exons 12–14 regions of PKP2 cDNA were amplified with two primers: exon 12-F: CTCCTCCGTATTTTTCC and exon 14-R: GTGGCCGTTATCTACTGTTGT. PCR products were directly cloned into bacteria using the TOPO TA Cloning Kit (Invitrogen) and sequenced using T7 primers at SBMRI DNA analysis facility.

Bisulphite sequencing. Genomic DNA purification from ≥2 × 10⁶ cells and bisulphite conversion of purified DNA was performed using the EZ DNA Methylation Direct Kit (Zymo Research) according to the manufacturer’s instructions. The NANOg promoter region of converted DNA was amplified by PCR using primers: NANOg-1F: AGAGATAGGAGGGTAAGTTTTTTT and NANOg-1R: ACTCT CACCAAAATACATTATTC, PCR products were directly cloned into bacteria and sequenced as mentioned above.

Gene microarray analysis. Total RNA was extracted from ≤10 cultured fibroblasts, or 5–10 undifferentiated hESCs or iPSCs. Sample amplification, labelling, and hybridization on Illumina Human HT-12v4 arrays were performed for all arrays using Illumina BeadStations in the SBMRI and Scripps Research Institute Microarray Core facilities according to the manufacturer’s instructions (http://www.illumina.com). Quality control of raw data was performed with the GenomeStudio software (Illumina) and analysed with various software detailed in the Supplementary Information. For the heat map, values across each probe were standardized to have mean 0 and standard deviation (s.d.) 1, and then plotted on a colour scale where green denotes negative s.d., black, 0; and red, positive s.d. The heat map showed close hierarchical clustering between hESCs and iPSCs based on 5,559 genes with at least twofold changes in mRNA expression levels when compared to the average levels of three fibroblast lines.

Lentiviral construction and viral transduction. Wild-type (WT) PKP2 cDNA was first cloned into pcDNA3.1 plasmid and the Xba1–EcoRI fragment containing WT PKP2 cDNA was then sub-cloned into pCDH-CMV-MCS-EF1-GFP lentivirus (System Biosciences). System Biosciences lentipackaging and concentration kit was used for lentiviral production following manufacturer’s instructions. We used the same protocol to generate lentiviruses containing control GFP and pRex1:Blasticidin/α-MHC-puromycin (Addgene).

Undifferentiated mutant PKP2 iPSCs with α-MHC-puromycin resistance were generated according to the detailed protocol published previously14,15. To generate mutant PKP2 iPSC-CMs with overexpression of control GFP or WT PKP2-GFP, we sub-cloned cDNA from iPSCs containing 10,000 cells per well in 5F-media containing 13-HODE, β-catenin luciferase assay. Production of lentiviruses containing 7XTCf-FLuc/5SV40-Puro (Addgene) is the same as described above. At 72 h after transduction, cells were replated at 2,000 cells per well for 6 h, then treated with media containing no BIO or 2.5 mM BIO for 16 h, and subsequently Luciferase assay was performed with Promega Luciferase assay system (catalogue no. PRE4030) according to manufacturer’s instructions. BIO treatment was used to assess the efficacy of the Luciferase assay and we used GloMax-96 Microplate Luminesometer (Promega) to measure luminescent values.

Immunocytochemistry, TUNEL count and lipid-laden cardiomyocyte count. We prepared 8-μm cryosections of EBs or CSs, and performed immunostaining according to standard protocols published previously4,14. Primary antibodies used were listed in Supplementary Table 3. Alexa Fluor 488 and Alexa Fluor 594 anti-mouse or anti-rabbit IgG (Invitrogen) were the secondary antibodies used for the fluorescence imaging. Samples were imaged on a deconvolution microscope (Observer.Z1, Zeiss) using the SlideBook software (Intelligent Imaging Innovations).

TUNEL staining with in situ Cell Death Detection Kit, TMR Red (Roche) and nuclei were counterstained with DAPI (10 μg ml⁻¹). Samples were conducted during the secondary antibody incubation period according to the manufacturer’s instructions. TUNEL and Nile Red staining were co-stained with one cardiac marker (anti-CNTI or α-actinin antibodies) so that cardiomyocyte-specific apoptosis and lipid-production could be counted. Cryosections with Nile Red staining were magnified using
SlideBook so as to count the number of cardiomyocytes that contained any cytoplasmic, round lipid droplets as the lipid-laden cardiomyocytes. Supplementary Table 4 summarized all clones of each type of iPSCs used for various experiments.

**Ca\textsuperscript{2+} imaging and curve fitting.** Puromycin-purified CSs were manually dissected to small clusters and clusters with slow beating rates (see Supplementary Fig. 16 for details) with or without pathogenic induction at approximately 60-day-old were loaded with Fura-2 AM (Molecular Probe). Ca\textsuperscript{2+} imaging was conducted with an inverted microscope (IX71, Olympus) and a cooled CCD camera (Hamamatsu) controlled by SlideBook. The \([\text{Ca}^{2+}]\text{\textsubscript{i}}\) was calculated from fluorescence ratio at 500 nm after excitation at 340 and 380 nm with a LAMBDA DG-4 system (Sutter Instrument) according to the standard method\textsuperscript{35}. CSs were externally paced at 1 Hz with 8 V/8 ms stimulus strength, and the relaxation phase of \([\text{Ca}^{2+}]\text{\textsubscript{i}}\) signals of the last paced beat was analysed with the SigmaPlot software (SPSS) using the Marquardt-Levenberg algorithm to obtain the relaxation time constant (\(\tau\)) of the best single-exponential curve fit. These CSs were then perfused with extra-cellular DMEM containing 5.8 mM \(\text{Ca}^{2+}\) media for 10 min to reach steady-state\textsuperscript{14}. The \(\tau\) after a similar pacing protocol was then obtained for comparison. All experiments were conducted at 37\textdegree C with all extracellular solutions pre-oxygenated with 95% O\textsubscript{2}/5% CO\textsubscript{2}. More than three sets of experiments were performed to construct Supplementary Fig. 13.

**Seahorse metabolic assay.** We used a Seahorse XF96 Extracellular Flux Analyzer\textsuperscript{25} to measure oxygen consumption rate (OCR, pmol min\textsuperscript{-1}) and extracellular acidification rate (ECAR, the H\textsuperscript{+} production rate, mP\textsubscript{H} min\textsuperscript{-1}) of JK mutant PKP2-iPSC-CMs and normal hS-iPSC-CMs in XF96 well plates according to Installation and Operation Manual from Seahorse Bioscience. XF assay media contain unbuffered DMEM, 5.5 mM glucose, and 0.5 mM carnitine. We used Etomoxir (ETO, 100 \mu M), a specific CPT-1 inhibitor, to assess the degree of fatty acid oxidation, and 2-deoxyglucose (2-DG, 50 mM), a blocker of glycolysis and pyruvate oxidation, to assess glucose utilization\textsuperscript{36}.

**Intracellular recording.** Standard intracellular recording of action potentials was performed as described previously\textsuperscript{14}.

**Statistical analysis.** Data were presented as the mean \(\pm\) s.e.m. Using the StatView/JMP program (SAS institute), statistical difference was analysed by an ANOVA procedure with post-hoc Tukey-Kramer test for multiple comparisons and by the unpaired \(t\)-test for pair-wise comparisons. A difference with a \(P\)-value \(< 0.05\) was considered statistically significant and was labelled with an asterisk (*) in all figures.

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