Modeling of Arrhythmogenic Right Ventricular Cardiomyopathy with

Human Induced Pluripotent Stem Cells

Running title: Caspi et al; hiPSCs modeling of ARVC

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Abstract:

**Background** - Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary heart muscle disorder resulting from desmosomal protein mutations. ARVC is characterized pathologically by fibrofatty infiltration and clinically by arrhythmias and sudden cardiac death. We aimed to establish a patient/disease-specific human induced pluripotent stem cells (hiPSCs) model of ARVC.

**Methods and Results** - Dermal fibroblasts were obtained from two ARVC patients with plakophilin-2 (PKP2) mutations, reprogrammed to generate hiPSCs, coaxed to differentiate into cardiomyocytes and then compared with healthy-control hiPSC-derived cardiomyocytes (hiPSCs-CMs). Real-time PCR showed significant decrease in the expression of PKP2 in the ARVC-hiPSCs-CMs. Immunostainings revealed reduced densities of PKP2, the associated desmosomal protein plakoglobin, and the gap-junction protein connexin-43. Electrophysiological assessment demonstrated prolonged field potential rise time in the ARVC-hiPSCs-CMs. Transmission electron microscopy identified widened and distorted desmosomes in the ARVC-hiPSCs-CMs. Clusters of lipid-droplets were identified in the ARVC-cardiomyocytes that displayed the more severe desmosomal pathology. This finding was associated with upregulation of the pro-adipogenic transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ). Exposure of the cells to adipogenic stimuli augmented desmosomal distortion and lipid accumulation. The latter phenomenon was prevented by application of a specific inhibitor of GSK-3β (BIO).

**Conclusions** - This study highlights the unique potential of the hiPSCs technology for modeling inherited cardiac disorders in general and ARVC specifically. The hiPSCs-CMs were demonstrated to recapitulate the ARVC phenotype in the dish, to provide mechanistic insights into early disease pathogenesis, and to provide a unique platform for drug discovery and testing in this disorder.

**Keywords**: arrhythmogenic right ventricular cardiomyopathy, stem cell, model, cardiomyocyte, lipids
Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heritable primary cardiac muscle disorder characterized by the replacement of cardiomyocytes, primarily in the right ventricle (RV), by fibrofatty tissue\textsuperscript{1,2}. The resulting disruption of normal myocardial architecture can lead to RV dysfunction, life-threatening arrhythmias and sudden cardiac death. Causative mutations have been identified in a significant number of patients, mainly in genes encoding for proteins associated with mechanical cell junctions (plakoglobin, plakophilin, desmoglein, desmocollin, and desmoplakin)\textsuperscript{3,4}. Mutated gene products were shown to be involved in remodeling of the intercalated disk.

Significant insights into ARVC pathogenesis have been gained in the last decade from several clinical, genetic, and pathological studies in ARVC patients\textsuperscript{5} and from relevant mouse models\textsuperscript{6}. Nevertheless, among the hurdles in studying the disease process has been the inability to study viable human cardiomyocytes from ARVC patients. The introduction of the induced pluripotent stem cells (iPSCs) technology\textsuperscript{7} may provide a possible solution to this challenge. The human iPSCs (hiPSCs) approach allows reprogramming of adult somatic cells into pluripotent stem cells by expression of a set of transcription factors\textsuperscript{8}. Recent studies demonstrated the robust ability to generate hiPSCs and to coax their differentiation into the cardiac lineage\textsuperscript{9,10}. More recent studies showed the ability to establish disease/patient-specific hiPSCs that can model disease-specific abnormalities in a number of inherited cardiac disorders such as different variants of the long QT syndrome\textsuperscript{11,12}, dilated and hypertrophic cardiomyopathies\textsuperscript{13,14}, and cathecolaminergic polymorphic ventricular tachycardia\textsuperscript{15}.

In the current study, we aimed to utilize the emerging hiPSCs technology to establish an \textit{in-vitro} model of ARVC. We hypothesized that cardiomyocytes differentiating from the ARVC
hiPSCs will recapitulate the disease phenotype and that investigating of these cells may provide new insights into the disease process.

**Materials and Methods**

**Supplementary methods**

An expanded description of the methods used for genomic sequencing, teratoma formation, karyotype analysis, immunostainings, TUNEL staining, gene expression analysis, microelectrode array (MEA) recordings and transmission electron microscopy is provided in the on-line supplementary information.

**Establishment and cardiomyocyte differentiation of the ARVC-hiPSCs**

All studies were approved by the Helsinki committee of Rambam Medical Center, Haifa, Israel. Dermal fibroblasts were isolated from a small skin biopsy, obtained following the patients' informed consent. The hiPSCs were established by retroviral delivery of Oct4, Sox-2, and Klf-4 followed by valproic-acid treatment as described\textsuperscript{11,16}. Several ARVC-hiPSCs clones, which were positively-stained with vital Tra-I-60 staining, were selected and expanded. Undifferentiated hiPSCs colonies were cultured on MEFs as previously described\textsuperscript{11,16}. Culture medium consisted of 80% knockout high-glucose glutamine-free DMEM with sodium-pyruvate supplemented with 20% serum-replacement, L-glutamine-1mM, mercaptoethanol-0.1mM, bFGF-4ng/mL and 1%-nonessential amino acids (Invitrogen).

Cardiomyocyte differentiation of the ARVC and healthy-control hiPSCs was induced using the embryoid body (EB) differentiating system\textsuperscript{17}. Briefly, undifferentiated hiPSCs were dispersed into small cell-clumps using collagenase-IV (300u/ml for 45min, Life-technologies) and cultivated in suspension for 10d as EBs. The EBs were plated on 0.1%-gelatin-coated culture
dishes. Spontaneously contracting area were microdissected and used for phenotypic characterization.

**Adipogenic medium**

In some of the studies the hiPSCs-CMs were cultured in an adipogenic promoting medium. Adipogenesis was promoted in a similar manner to that reported previously\(^1\) with slight modifications. Briefly, microdissected control and ARVC-hiPSC-CMs (at 30d of differentiation) were treated with 20% fetal-bovine-serum containing media supplemented with 10\(\mu\)g/ml insulin (Biological-Industries, Israel), 0.5mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 2.5\(\mu\)M dexamethasone (Sigma-Aldrich) and StemPro LipoMax\(^2\) (1:200) for 3d. Fresh media, containing 20% FBS supplemented with insulin (10\(\mu\)g/ml) and StemPro-LipoMax (1:200) was then replaced every other day for additional 14d. In the experiments evaluating GSK-3\(\beta\) inhibition, 10\(\mu\)M of 6-bromoindirubin-3’-oxime (BIO, Sigma-Aldrich) was added.

**Statistical analysis**

Continuous variables are reported as mean±SEM. Categorical variables are expressed as frequencies. Differences between group means were compared using the Mann-Whitney U test (except for differences between drug treatment and baseline in which Wilcoxon test was used). Comparison between lipid accumulation and desmosomal gap-width quartiles was conducted using Chi-Square for trend analysis. Receiver-operating-characteristic (ROC) curve analysis was used in order to assess the sensitivity and specificity of desmosomal gap width as a marker and a predictor of lipid droplet accumulation. To this aim, desmosomal gap width was used as a continuous variable (width measured in nm) and lipid droplet accumulation within the same cell was used as a categorical binary value. ROC curve analysis was conducted using SPSS-16. We calculated the area under the curve (AUC), also known as concordance statistic, to assess
discrimination. A value of p<0.05 was considered statistically significant.

Results

Generation of the ARVC patient-specific hiPSCs

Dermal fibroblasts were obtained from a 30-year-old male, diagnosed previously with ARVC according to the 2010 task force criteria for ARVC. Clinically, the patient presented with episodes of rapid palpitations, near-syncope, and a family history of sudden cardiac death. His baseline electrocardiogram revealed typical T wave inversions in leads V1-V3 (Fig.1A). Episodes of ventricular tachycardia were also recorded, displaying a left-bundle-branch-block like morphology (Fig.1B). Echocardiographic examination and RV ventriculography revealed a dilated and trabeculated RV with reduced global RV function and segmental wall motion abnormalities. Electroanatomical mapping of the RV identified large areas with reduced voltage (Fig.1C), a finding previously shown to correlate with the diseased RV regions.

Genetic analysis identified a heterozygous insertion of T in position 972 in exon 3 of the plakophilin-2 (PKP2) gene (c.972InsT/N) resulting in a frame shift from amino-acid 324 and to a stop codon in position 335 (A324fs335X). This mutation has not been previously reported. The patient's fibroblasts were reprogrammed to generate the patient-specific ARVC-hiPSCs by retroviral delivery of Oct3/4, Sox2, and Klf4. Similar hiPSCs previously created from fibroblasts of a healthy individual using an identical reprogramming strategy served as controls.

Sequencing of the affected allele (Fig.1D) verified that the PKP2 mutation was present in the ARVC-hiPSCs colonies but not in healthy-control cells.

The ARVC-hiPSCs colonies displayed typical human embryonic stem cells morphology (Fig.2A, left-panel), were stained positively for the pluripotent markers Oct-4, NANOG, SSEA-4 and Tra-1-60 (Fig.2A), displayed alkaline-phosphatase activity (Fig.2B), and maintained a
normal karyotype. Pluripotency of the ARVC-hiPSCs was verified by the presence of cell-derivatives of all three germ layers in in-vitro differentiating EBs, as manifested by the positive staining for Desmin (mesoderm), α-fetoprotein (endoderm) and Nestin (ectoderm) (Fig.2C).

Similarly, injection of undifferentiated ARVC-hiPSCs into SCID-beige mice led to the formation of teratomas containing advanced tissue-derivatives of all three germ layers (Fig.2D). Finally, ARVC-hiPSCs showed silencing of the three retroviral transgenes (Fig.2E) and reactivation of endogenous pluripotency genes (NANOG, FOXD3 and OCT4) (Fig.2F), indicating successful reprogramming.

**Cardiomyocyte differentiation**

The EB differentiation system was used to coax the differentiation of the hiPSCs into the cardiac lineage (Supp. Movie 1). Real-time quantitative PCR analysis showed comparable levels of expression of cardiac-specific genes in the ARVC and healthy-control hiPSCs-derived cardiomyocytes (hiPSCs-CMs) and minimal expression of these genes in the undifferentiated cells (Fig.3A). Similarly, immunostainings for the sarcomeric proteins cardiac troponin I (cTnI) and α-actinin (Fig.3B) confirmed the cardiomyocyte phenotype of the differentiating hiPSCs-CMs.

Extracellular multielectrode array (MEA) recordings established the presence of cardiac-specific electrical activity by the ARVC-hiPSCs-CMs (Fig.3C) and the development of a functional syncytium with pacemaker activity and action-potential propagation (Fig.3D and Supp. Movie 2). Adequate chronotropic responses to adrenergic and cholinergic stimuli were noted following application of 10μM isoproterenol (increase in beating frequency by 156±55%, p<0.05, n=5) and 1μM carbamylcholine (decrease in beating frequency by 45±12%, p<0.05, n=5).
Automated analysis of the field-potential rise-time, previously suggested as a surrogate marker for the cellular action-potential rise-time\textsuperscript{24}, revealed significant prolongation of this parameter in the ARVC-hiPSCs-CMs when compared to healthy-control cells (11.5±2.6ms vs. 4.1±1.3ms, respectively, \(p<0.05\), Fig.3E). Finally, analysis of the corrected field-potential duration (cFPD) revealed a slightly prolonged cFPD in ARVC-hiPSCs-CMs, which was not statistically significant when compared to healthy-control cells (0.36±0.02 vs. 0.42±0.03).

**Immunostaining for desmosomal and gap-junction proteins**

Since the mutations associated with ARVC involve mainly desmosomal proteins, we initially performed real-time quantitative PCR analysis evaluating the expression of the relevant genes in ARVC and healthy-control hiPSCs-CMs. These studies revealed a significant reduction in the expression of \(PKP2\) in the ARVC-cardiomyocytes when compared to healthy-control cells (\(p<0.01\)). In contrast, no significant reductions were noted in the expression of the associated desmosomal genes \(JUP\) (plakoglobin) and \(DSP\) (desmoplakin) (Fig.4A).

We next performed immunostainings, targeting the desmosomal proteins in small cell-clusters of both healthy-control and ARVC hiPSCs-CMs (Fig.4B). We initially focused on the evaluation of the staining pattern of \(PKP2\) (Fig.4B; top-panels). Notice the typical membranous punctuate staining pattern for \(PKP2\) in the healthy-control hiPSCs-CMs (Fig.4B; top, left-panel) and the diminished \(PKP2\) immunosignal in the ARVC-cardiomyocytes (Fig.4B; top, right-panel). Thus, as expected from the nature of the \(PKP2\) mutation in the ARVC patient, quantitative analysis revealed a significant reduction in the density of the \(PKP2\) immunosignal (expressed as percentage of the total cardiomyocyte cellular area) in the ARVC-hiPSCs-CMs when compared to healthy-control cells (2.2±0.6% vs. 9.5±3.6%, \(p<0.05\)) (Fig.4C, top-panel).

In a similar manner, immunostaining analysis for the structurally-associated desmosomal
protein plakoglobin (also known as γ-catenin) revealed a significant reduction (p<0.05) in the immunofluorescent signal area-density in the ARVC (1.0±0.1%) when compared to healthy-control (7.4±0.9%) cardiomyocytes (Figs.4B-C, middle-panels). Finally, we also evaluated the impact of the PKP2 mutation on the associated intercalated-disc structure, gap-junction.

Interestingly, immunofluorescence analysis, targeting the major gap-junction protein connexin-43 (Cx43), revealed a significant reduction in the Cx43 immunofluorescent signal in the ARVC-hiPSCs-CMs when compared to healthy-control cells (3.4±0.9% vs. 0.9±0.4%; p<0.05; Figs.4B&C, bottom-panels).

**Ultrastructural analysis**

To further evaluate the impact of the PKP2 mutation on the ultrastructural properties of the differentiated ARVC-hiPSCS-CMs, we performed transmission electron microscopy analysis of the ARVC and healthy-control cells at 40d of differentiation. Both control and ARVC cardiomyocytes exhibited large quantities of myofibrils organized into typical sarcomeres by parallel Z bands (Figs.5A-B). Interestingly, in 33% (13 out of 39) of the ARVC-hiPSCs-CMs studied we could identify the presence of clusters of lipid-droplets accumulating within the cytoplasm (Figs.5B&E and 6B). Such a phenomenon was not observed in any of the healthy-control hiPSCs-CMs.

We next focused on the ultrastructural properties of the desmosomes in the hiPSCs-CMs and noted significant morphological differences between healthy-control (Figs.5A&C) and ARVC (Figs.5B, D-E) cardiomyocytes. This was manifested by the presence of variable degrees of structural distortion of desmosomes in the ARVC-cardiomyocytes but not in the healthy-control cells. Notice the hazy, pale and dissymmetric appearance of the ARVC desmosomes and the widened desmosomal gaps in the examples provided (Figs.5B,D-E). To quantify the degree
of desmosomal abnormalities in the ARVD-hiPSCs-CMs, we performed quantitative morphometric assessment of the desmosomal dimensions in both the ARVC and control cardiomyocytes (Figs.5C-D). This analysis revealed a significant increase in the desmosomal internal gap-width (32±2 vs. 24±1nm, p<0.05) and in the total external desmosomal width (171±12 vs. 101±5nm, p<0.01) in the ARVC-hiPSCs-CMs when compared to healthy-control cells (Figs.5F-G).

Strikingly, the degree of desmosomal abnormalities seemed to correlate with the accumulation of lipid-droplets within the ARVC-cardiomyocytes. This can be appreciated in Figs.5B&6B, where the presence of severe desmosomal distortion was associated with intracellular accumulation of clusters of lipid-droplets. In contrast, ARVC-cardiomyocytes showing minimal desmosomal abnormalities did not contain similar clusters (Fig.6A).

To further evaluate the relationship between desmosomal abnormalities and accumulation of lipid-droplets in the ARVC-hiPSCs-CMs, we graded the degree of desmosomal abnormalities in each cell using the average measured desmosomal gap-width within the cell. We then divided all cells into four quartiles based on this parameter and compared the percentage of cells containing lipid-droplets clusters in each group. As depicted in Fig.6C, lipid accumulation was correlated with desmosomal gap widening quartiles (p<0.05). To further assess whether desmosomal abnormalities can predict the presence of lipid accumulation within the cells, we generated a receiver operating characteristic (ROC) curve. Evaluation of the area under the ROC curve demonstrated a significant correlation between desmosomal gap widening and lipid accumulation (AUC= 0.84, 95% CI, 0.73 to 0.94, n=50) with an optimal cutoff value of 25.9nm (Fig.6D).

Finally, we also evaluated, using real-time PCR analysis, the expression level of the
master pro-adipogenic transcription factor PPAR-γ. This analysis revealed more than four times increase in the expression levels of PPAR-γ in the ARVC-hiPSCs-CMs when compared to healthy-control cells, suggesting the activation of the adipogenic signaling pathway in these cells (Fig.5H).

**Confirmation of the pathological findings in a second ARVC patient**

To evaluate whether the molecular and ultrastructural properties described above may be applicable to other ARVC patients carrying alternative *PKP2* mutations, we generated a second patient-specific hiPSCs line. Dermal fibroblasts were obtained from a 22-year-old ARVC patient displaying the characteristic RV pathology and ventricular arrhythmias (Suppl.Figs.1A-B).

Genetic analysis identified a heterozygous deletion of ACAG in position 148-151 in exon 1 of the *PKP2* gene in the defected allele (c.148_151delACAG/N) (Suppl.Fig.1C) resulting in replacement of Threonine by Serine in position 50 and in a frame shift leading to a stop codon in position 110 (p.T50SfsX110). A similar mutation has been previously reported in the literature in four ARVC patients.

The patient's fibroblasts were reprogrammed to generate ARVC-hiPSCs (ARVC-B) by retroviral delivery of Oct3/4, Sox2, and Klf4. The reprogrammed cells generated typical hiPSC colonies (Supp.Fig.2A), displayed alkaline-phosphatase activity (Supp.Fig.2B) and stained positively for the same pluripotent markers (Supp.Fig.2C). Pluripotency of the ARVC-B hiPSCs was verified by the presence of cell-derivatives of all three germ layers in in-vitro differentiating EBs (Suppl.Fig.2D). The ARVC-B hiPSCs also showed silencing of the three retroviral transgenes (Suppl.Fig.2E) and reactivation of endogenous pluripotency genes (Supp.Fig.2F), indicating successful reprogramming.

Cardiomyocytes were then derived from the ARVC-B hiPSCs, using the same EB-based
differentiating technique, and the generated beating areas were also stained positively for cTnI (Supp.Fig.3A). Consistent with our finding in the first patient, real-time PCR analysis demonstrated significant reduction in the gene expression levels of PKP2 ($p<0.05$) in the ARVC-B hiPSCs-CMs when compared to healthy-control cells, while showing similar levels of expression of JUP and DSP (Supp.Fig.3B). Similarly, immunostaining analysis of the ARVC-B hiPSC-derived cardiac-tissue revealed reduced levels of Plakophilin-2 (Supp.Fig.3A). This was coupled with a reduction in the immunosignals of plakoglobin and Cx43 when compared to control cells (Supp.Figs.3D-E).

Finally, electron microscopy analysis identified marked desmosomal abnormalities also in ARVC-B hiPSCs-CMs (Suppl.Figs.4A-B) and the accumulation of lipid-droplets in several of the diseased cells (Suppl.Figs.4A&C). Notice again in the example provided (Supp.Fig.4D) that the cells containing the distorted desmosomes (characterized by increased desmosomal widths) were also characterized by accumulation of lipid-droplets clusters, recapitulating the findings in the first ARVC (c.972InsT) patient.

**Vulnerability to apoptosis**

Apoptosis has been proposed as one of the mechanisms mediating cardiomyocyte loss in ARVC in both *in-vitro*\(^{18}\) and *in-vivo*\(^{26}\) studies. To assess for the presence of apoptosis, we carried out TUNEL analysis of both ARVC and healthy-control hiPSCs-CMs following exposure to serum starvation, a well-known stimulator of apoptosis. The results of these studies revealed a significantly higher percentage of apoptotic cells in the ARVC-hiPSCs-CMs (3.8±1%, \(n=7\), \(p<0.05\), Supp.Fig.5) when compared to healthy-control cells (1.0±0.6%).

**The impact of adipogenic stimulus**

Cardiomyocytes derived from ARVC animal models were suggested to display a tendency
toward adipogenesis\textsuperscript{18}. In order to evaluate whether ARVC-hiPSCs-CMs recapitulate such a propensity, we exposed healthy-control and ARVC hiPSCs-CMs to a combination of 3-isobutyl-1-methylxanthine, dexamethasone and insulin in conjunction with lipid-supplementation (MDI-Lipo). Treated healthy-control cells exhibited minimal or no lipid-droplets accumulation with preserved desmosomal and sarcomeric structures (Fig.7A). In contrast, the treated ARVC-hiPSCs-CMs displayed extensive lipid-droplets accumulation throughout most of the cytoplasm. Interestingly, this lipid accumulation was associated with paucity of desmosomal structures (Fig.7B).

Finally, we also evaluated whether the accumulation of lipid-droplets in the ARVC-cardiomyocytes following the adipogenic medium challenge could be prevented. Since previous studies implied that reduced canonical Wnt pathway signaling may be associated with the ARVC phenotype and lipid-droplet accumulation\textsuperscript{18}, we aimed to determine the activity level of this pathway in the ARVC-hiPSCs-CMs. To this end we analyzed, in both ARVC and control hiPSCs-CMs cardiomyocytes, the nuclear density of \(\beta\)-catenin, an immunostaining assay previously suggested to serve as a surrogate marker for canonical Wnt pathway activity\textsuperscript{27}. Our results (Supp.Fig.6) revealed a significant reduction in the nuclear density of \(\beta\)-catenin in the ARVC-hiPSC-CMs (5.2\%\pm1.5\%) when compared to healthy-control cardiomyocytes (12.1\%\pm1.8\%, \(p<0.05\), \(n=7\)), suggesting reduced Wnt activity in the former cells.

Next, to evaluate whether activation of the canonical Wnt pathway can reverse the lipid droplet accumulation, we treated the ARVC-hiPSCs-CMs simultaneous with the adipogenic media with BIO (10\(\mu\)M), a specific inhibitor of GSK-3\(\beta\). This compound, a known activator of the canonical Wnt pathway, was previously shown to prevent lipid accumulation \textit{in-vitro} in cardiac progenitor cells derived from an ARVC mouse model\textsuperscript{28}.
Application of BIO did not affect sarcomeric organization in the ARVC-cardiomyocytes and did not seem to significantly alter desmosomal abnormalities in these cells. Thus, quantitative ultrastructural analysis revealed that both desmosomal internal gap-width (36±2 vs. 39±5nm) and external desmosomal width (169±21 vs. 214±34nm) did not significantly change following the addition of BIO to the adipogenic medium (Fig. 7C). Similarly, a comparable low number of desmosomal structures were found in the ARVC-cardiomyocytes treated with the adipogenic medium with or without simultaneous exposure to BIO (0.9±0.2 vs. 1.4±0.6 desmosomes/cell). Importantly, in contrast to the lack of significant effects of BIO on the desmosomes, its application had a dramatic effect in preventing intracellular lipid-droplets build-up in the ARVC-hiPSCs-CMs as can be appreciated in the example in Fig. 7D.

**Discussion**

In the current study we evaluated the ability of the hiPSCs technology to model ARVC and to provide mechanistic insights into the human disease process. Patient-specific hiPSCs were created from two patients with a clinical diagnosis of ARVC and genetic evidence for *PKP2* mutations.

By studying cardiomyocytes differentiating from the ARVC-hiPSCs and comparing them to healthy-control cells, we were able to: (1) identify significant desmosomal abnormalities in the ARVC-hiPSCs-CMs in both the electron microscopy studies and in the PKP2 and plakoglobin immunostaining analysis; (2) demonstrate the effect of desmosomal remodeling on the associated intercalated disc structure gap-junction; (3) reveal the accumulation of lipid-droplets in many of the ARVC-cardiomyocytes and the up-regulation of the pro-adipogenic transcription factor PPAR-γ; (4) identify a clear correlation between the degree of desmosomal abnormalities in the ARVC-cardiomyocytes and the probability for lipid accumulation in the same cells; (5)
show that ARVC cardiomyocytes have an increased vulnerability to apoptosis; and (6) demonstrate the ability of lipogenic stress to worsen the ARVC phenotype in terms of lipid-droplet accumulation and the ability of the small molecule BIO (an inhibitor of GSK-3β) to suppress the latter effect.

A major breakthrough in the understanding of ARVC came with the realization that this syndrome is associated with mutations in desmosomal proteins. Besides confirming the genetic nature of the disease (autosomal dominant inheritance with variable penetrance) this discovery fueled several studies attempting to uncover the relationship between such mutations, the resulting desmosomal structural abnormalities, and the eventual ARVC pathological and clinical findings. Consistent with these findings, we also identified in the two patients studied (both presenting with a highly arrhythmogenic phenotype and advanced RV pathology) mutations in the PKP2 gene. Both the novel mutation identified in our first patient (c.972InsT) and the previously reported mutation identified in the second patient (c.148–151delACAG/N) resulted in an early stop-codon. As expected from the nature of such mutations (leading to a truncated protein) these allelic derangements resulted in a significant reduction in the density of the PKP2 immunosignal in the ARVC-hiPSCs-CMs. Interestingly, we could also identify a reduction in the mRNA levels of PKP2 in the ARVC-hiPSCs-CMs. This finding most probably results from a nonsense-mediated mRNA decay mechanism.

Importantly, in both patients, the in-vitro ARVC-hiPSCs-CMs model was able to recapitulate the abnormal desmosomal phenotype, thought to be at the center of ARVC pathogenesis. This was manifested in the ultrastructural analysis of the ARVC-hiPSCs-CMs by the presence of significant desmosomal abnormalities resembling those previously reported in ARVC patients and murine models. Similarly, a significant reduction was noted in the
density of the immunosignals of PKP2 and the structurally-associated desmosomal protein plakoglobin in the ARVC-hiPSCs-CMs. The latter finding is in agreement with the work of Asimaki et al. who observed a significant reduction in the immunosignal of both proteins in endomyocardial biopsies of ARVC patients\textsuperscript{34} and with a recent study using a similar ARVC-hiPSCs model\textsuperscript{35}.

In healthy myocardium, plakoglobin is found mainly at junctional plaques and the cytoplasmic concentration of plakoglobin is kept low due to proteasomal degradation. In ARVC, the plakoglobin signal level at junctional plaques is diffusely reduced\textsuperscript{34,36} and plakoglobin is thought to accumulate in the cytosolic pool, from which it can diffuse into the nucleus and alter gene expression\textsuperscript{17}. The translocation of plakoglobin to the cytosolic and nuclear pools is thought to result in an attenuated spatial concentration at the desmosomes, translating to the reduced immunosignal identified in the current and previous studies\textsuperscript{34,36} at these membranous structures. In this regards it is interesting to note that our real-time PCR data revealed an unaltered expression of plakoglobin at the mRNA level in both patients despite the observed decrease of its membranous immunosignal levels, a finding that also supports the aforementioned hypothesis of desmosomal disintegration leading to cytoplasmatic translocation of plakoglobin.

Recent studies suggest that abnormalities in the desmosomal proteins in ARVC may also affect other related components of the intercalated disc, including gap-junctions and sodium channels\textsuperscript{29,38}. Supporting these previous observations, our results show a significant reduction in gap-junction density (as suggested from the Cx43 immunofluorescent analysis) in the ARVC-hiPSCs-CMs when compared to healthy-control cells. To evaluate whether these structural findings translate into functional derangements, we quantified the field-potential rise-time in both ARVC and control cardiomyocytes. Halbach et al.\textsuperscript{24}, utilizing simultaneous microelectrode
extracellular recordings and intracellular action-potential measurements from embryonic cardiomyocytes, previously demonstrated that the field potential rise-time highly correlates with action-potential rise time and that reduced excitability and conduction can be identified by prolongation of this parameter. Consequentially, the presence of significantly prolonged field potential rise-time in the ARVC-hiPSCs-CMs suggests reduced excitability or conduction slowing in these cells and may contribute to the increase arrhythmogenesis observed in ARVC patients, even prior to the development of significant ventricular structural alterations.

Another important finding of the current study relates to the identification of clusters of lipid-droplets within many (approximately third) of the ARVC-hiPSCs-CMs. Consistent with this finding we also observed a significant increase in the expression of the master pro-adipogenic transcription PPAR-γ in the ARVC-cardiomyocytes. Moreover, we were able to demonstrate a functional pro-adipogenic state of the ARVC-cardiomyocytes, as suggested by the robust lipid accumulation following exposure to a lipogenic media. The latter finding is in agreement with the recent paper of Kim et al. 39, which also found that ARVC-hiPSC-CMs (derived from ARVC patients with different PKP2 mutations) are prone to lipid accumulation following treatment with various adipogenic stimuli 39. While lipid accumulation is considered one of the hallmarks of ARVC, there is uncertainty in whether this is due to intramyocardial lipid accumulation or due to fibrofatty replacement of necrotic myocardial tissue. Intra-cardiomyocyte lipid accumulation was previously shown only in rodent models 3,18,40 and in a few human myocardial specimens analyzed by electron microscopy 41,42.

Finally, the results of our study imply that a pro-adipogenic state of the diseased cardiomyocytes resulting in intramyocardial lipid accumulation may have at least a partial role in ARVC disease pathogenesis. Interestingly, the effect of the adipogenic stimuli on the ARVC-
cardiomyocytes could be prevented by activation of the canonical Wnt pathway in these cells by the small molecule BIO, a specific inhibitor of GSK-3β. This effect may be explained by the inherent reduced canonical Wnt pathway signaling in the ARVC-cardiomyocytes as demonstrated by the reduced density of nuclear β-catenin in our study. Additionally, these results are in agreement with the work of Lombardi et al. who demonstrated that a similar treatment could prevent lipid accumulation in cardiac progenitor cells derived from a mouse model of ARVC\textsuperscript{28}.

One of the key novel findings of the current study was the fact that intracellular lipid-droplets accumulation could be predicted based on the degree of desmosomal abnormalities in the same cell. The relationship between the mutations in desmosomal genes, desmosomal structural abnormalities, and the resulting fibro-fatty myocardial phenotype in ARVC has been a long-standing enigma of the disease pathogenesis. A potential mechanism that may explain the tight relationship identified in our study between desmosomal abnormalities and lipid accumulation may be derived from recent findings by the Marian’s group\textsuperscript{18,28,43}. These studies suggest a crosstalk between desmosomal disintegration and a pro-adipogenic state. Plakoglobin (γ-catenin) was pinpointed as a key mediator of this crosstalk, dislodging from the disintegrated abnormal desmosomes and inhibiting the canonical Wnt pathway due to its resemblance to β-catenin, consequentially promoting a pro-adipogenic state\textsuperscript{18}.

Despite the unique advantages of the hiPSCs approach for modeling inherited cardiac disorders in general and ARVC specifically, as highlighted in this study, this strategy still possesses some inherent limitations such as the inability to study pathological phenomena at the whole-organ and systemic levels. This may be important, for example, for studying the potential mechanistic role of inflammation in ARVD\textsuperscript{44}. Similarly, other factors that may play a role in
ARVC pathogenesis such as multicellular interactions, the three-dimensional properties of the
tissue, and external mechanical forces cannot be modeled currently using the hiPSCs
cardiomyocyte system. Further advancements in the field of in-vitro cardiac tissue-engineering,
however, may provide a possible solution to these challenges. An additional limitation stems
from the relatively early-stage phenotype of the hiPSCs-CMs, which may not adequately
represent the adult pathology. In this regards, it is interesting to note that recent studies suggested
a potential role for early-differentiating cardiac cells in ARVC pathogenesis. In addition,
significant efforts are currently made in the field by several groups in an attempt to devise
strategies to induce hiPSCs-CMs maturation.

Despite the aforementioned limitations, our data demonstrates the unique ability of the
hiPSCs technology to model ARVC. Using this approach we showed that some of the key
features of ARVC, namely desmosomal-related pathology and lipid accumulation can be
identified in the ARVC-hiPSCs-CMs. This study also provides important mechanistic insights
into the cardiac pathology in ARVC as well as confirming some of the existing theories in
patient-specific human tissues. Finally, the generated model-system provides a unique platform
to evaluate existing as well as novel therapeutic strategies for ARVC.

Acknowledgments: We thank Dr. Doron Aronson for his statistical advice, Dr. Edith Suss-Toby
for her help in imaging, and Ira Minkov and Sharona Avital for their help in electron-microscopy
analysis.

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[ERC-2010-StG-260830-Cardio-iPS].

Conflict of Interest Disclosures: None
References:


Figure Legends:

Figure 1: Clinical characteristics of the ARVC patient. [A] Twelve lead electrocardiogram of the ARVC patient showing inverted T-waves in leads V1-V3. [B] Electrocardiographic tracing showing the development of ventricular tachycardia in the ARVC patient. [C] Right anterior oblique view of the electro-anatomical unipolar voltage map of the RV of the ARVC patient.
Note the extensive area of low-voltage (red indicates unipolar electrograms<3mV), corresponding to the diseased regions in the RV inflow, outflow, and anterolateral free-wall. [D] Sequencing of the mutated allele of the plakophilin-2 (PKP2) gene in the ARVC-hiPSCs confirming the insertion of T in position 972 in exon 3 of the PKP2 gene (c.972InsT), which results in a stop codon in position 335 (A324fs335X/N).

**Figure 2:** Derivation and characterization of the ARVC-hiPSCs. [A] Phase-contrast (left) and immunofluorescent staining of the ARVC-hiPSCs colonies for the pluripotency markers NANOG, OCT4, SSEA4, and TRA-1-60. [B] Positive staining of the ARVC-hiPSCs colonies for alkaline-phosphatase. [C] Immunostaining of in-vitro differentiating EBs for Desmin (mesoderm), alpha-Fetoprotein (AFP, endoderm) and Nestin (ectoderm). [D] Teratoma formation in SCID mice following injection of undifferentiated ARVC-hiPSCs. Note the presence of hyaline-cartilage (mesoderm), columnar-lining epithelium (endoderm), and melanocytes (ectoderm). Scale-bars for A-D:50μm. [E] Real-time quantitative PCR showing the down-regulation of OCT3/4, KLF4 and SOX2 transgenes in the ARVC-hiPSCs (in two clones: AR-hiPS-1 and AR-hiPS-2). Values are normalized to the house-keeping gene RPL-7 and expressed as mean±SEM. Expression values (Relative Quantitation- RQ) are relative to HEK293T cells (HEK) transiently transfected with the three plasmids to produce reprogramming virions (n=3 biological repetitions with 3 technical replicates for each biological repetition). [F] Real-time quantitative PCR evaluating the endogenous levels of the pluripotency genes NANOG, FOXD3, and OCT 3/4 in healthy-control fibroblasts, ARVC fibroblasts, control-hiPSCs and ARVC-hiPSCs clones (1 and 2). Values are normalized to the house-keeping gene RPL-7 and
expressed as mean±SEM. Expression values (RQ-%) are relative to the levels in the patient's fibroblasts (n=3)

**Figure 3:** Cardiomyocyte differentiation. [A] Real-time PCR analysis showing comparable levels of expression of the cardiac-specific genes MYL2 (MLC-2v), MYH7 (β-MHC), MYH6 (α-MHC), TNNI3 (cTNI) and ISL-1 in cardiomyocytes differentiating from the ARVC and healthy-control hiPSCs. Note also the marked increase in these genes in both cardiomyocyte types when compared to the undifferentiated cells (n=3). [B] Immunostaining of differentiating ARVC-hiPSCs-CMs for the sarcomeric proteins α-actinin (green, left-panel) and cTnI (red, right). Bars-20μm. [C-D] MEA recordings from the ARVC hiPSCs-derived cardiac-tissue (C). This information is used to generate activation maps (D) showing activation propagating from the pacemaker area (red) to the rest of the tissue. [E] Field-potential rise-time analysis of extracellular recordings from the ARVC (red) and control (blue) hiPSCs-CMs. Note that the ARVC-hiPSCs-CMs displayed significant longer rise-times (*p<0.05, n=7).

**Figure 4:** Expression of desmosomal related proteins. [A] Real-time PCR analysis quantifying the expression of desmosomal-related genes. Note the significant reduction in the expression of PKP2 in the ARVC-hiPSC-CMs when compared to healthy-control cells (*p<0.05). No significant differences were noted, however, in the expression levels of JUP (Plakoglobin) and DSP (Desmoplakin). Values are normalized to the house-keeping gene RPL-7 and expressed as mean±SEM. Expression values (RQ%) are relative to levels in healthy-control cardiomyocytes. [B-C] Immunostainings of the healthy-control (left-panels) and ARVC (right-panels) hiPSCs-CMs targeting PKP2 (top), plakoglobin (middle), and Cx43 (bottom). [C] Quantitative
evaluation of the density of the PKP2 (n=8) (top), plakoglobin (n=9) (middle) and Cx43 (n=8) (bottom) immunosignals. Results are described relative to total cardiomyocyte area. Note the significant reduction in the densities of the immunosignals of both desmosomal and gap-junction proteins in the ARVC-hiPSCs-CMs (cardiomyocyte-clusters, *p<0.05).

Figure 5: Ultrastructural characterization. [A] Transmission electron-microscopy image of healthy-control hiPSCs-CMs. Note the presence of developing sarcomeres. The intercalated disc area is magnified in the insert and displays a normally-looking desmosome. [B] Transmission electron-microscopy image of the ARVC-hiPSCs-CMs. Note the presence of a structurally abnormal desmosome in the magnified area (insert). Also notice the presence of a cluster of lipid-droplets within the ARVC-cardiomyocyte. [C-D] Electron-microscopy images showing examples of desmosomes from healthy-control (C) and ARVC (D) hiPSCs-CMs. Also shown are the measurements made to analyze desmosomal gap-width (short white-line) and total desmosome width (long white-line). Note the presence of a structurally distorted desmosome in the ARVC-cardiomyocyte with a widened gap and hazy appearance. [E] Additional high-magnification images depicting distorted desmosomes in ARVC-hiPSCs-CMs.

Abbreviations: S-sarcomere, Nuc-nucleus, M-mitochondria, L-lipid droplets, D-desmosome. [F-G] Quantitative morphometric assessment of the desmosomal gap-width (F) and total desmosome width (G) dimensions. Note the significant increase in these parameters in the ARVC-hiPSCs-CMs (*p<0.05, n=57 and **p<0.01, n=57). [H] Real-time PCR analysis showing significant up-regulation of PPARG expression in ARVC-hiPSCs-CMs (**p<0.01, n=4). Values are normalized to the house-keeping gene RPL-7 and expressed as mean±SEM. Expression values (RQ%) are relative to levels in healthy-control cardiomyocytes.
Figure 6: Correlation between desmosomal abnormalities and lipid-droplet accumulation. [A-B] Transmission electron-microscopy images of a lipid-free (A) and a lipid-containing ARVC-hiPSCs-CM. Desmosomal distortion, asymmetry and a widened desmosomal gap (inserts) are noted in the lipid-containing cell. In contrast, relatively normal-looking and symmetric desmosome are seen in the lipid-free cell. S-sarcomere, Nuc-nucleus, M-mitochondria, G-glycogen, L-lipid-droplets, D-Desmosome. [C] Frequency of cells showing accumulation of lipid-droplets clusters as function of the desmosomal gap-widths (divided to quartiles). Note that cells in the higher quartiles of desmosomal gap-widths are associated with a significantly higher probability of containing lipid-droplets (*p<0.05, chi-square for trend-analysis.). [D] Receiver operating curve (ROC) depicting the sensitivity and specificity of desmosomal gap-width in predicting lipid-droplets accumulation (n=50).

Figure 7: Effect of pro-adipogenic stimulus. [A-B] Transmission electron-microscopy images of healthy-control (A) and ARVC (B) hiPSCs-CMs exposed to lipogenic media. Note the absence of lipid-droplets in control cells and the extensive lipid accumulation in the ARVC-hiPSCs-CMs, compromising the majority of the cell's cytoplasm. The lipid-clusters contained both low and high electron-dense lipid-droplets (known to correlate with the presence of saturated and unsaturated fatty acids, respectively). [C-D] Transmission electron-microscopy images of the ARVC-hiPSCs-CMs following simultaneous treatment with the lipogenic-media and BIO. Note that BIO application did not reverse the desmosomal abnormalities in the ARVC-hiPSCs-CMs (C) but significantly reduced lipid-droplets accumulation in treated cells (D). S-sarcomere myofibrils, Nuc-nucleus, M-mitochondria, L-lipid-droplets, D-Desmosome.
Figure 2

A

ARVC-hiPSC

Oct 4

Nanog

SSEA 4

TRA-1-60

B

C

Desmin (Mesoderm)

AFP (Endoderm)

Nestin (Ectoderm)

D

Mesoderm

Endoderm

Ectoderm
F  Desmosomal gap width

G  Total desmosome width

H  PPARG

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Control  ARVC  Control  ARVC  Control  ARVC

---
Modeling of Arrhythmogenic Right Ventricular Cardiomyopathy with Human Induced Pluripotent Stem Cells
Oren Caspi, Irit Huber, Amira Gepstein, Gil Arbel, Leonid Maizels, Monther Boulos and Lior Gepstein

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Genomic sequencing

Genomic DNA was isolated from the hiPSCs colonies using the high-pure PCR template preparation kit (Roche). The relevant DNA fragments of exon 3 of the plakophilin-2 (PKP2) gene were then amplified by PCR reaction using 100ng genomic DNA (primer sequences: forward TGACACATAACCACAGACAGTAC, reverse GTCACTGAAAGTGCTTCTCTCA). The PCR products were then sequenced.

Teratoma formation and karyotype analysis

Undifferentiated hiPSCs were injected subcutaneously to immunodeficient SCID-beige mice. Teratomas, developing 8 weeks after injection, were harvested, cryosectioned (10µm) and stained with hematoxylin and eosin (H&E). Karyotype analysis was performed by the institution’s cytogenetic laboratory according to standard procedures.

Immunostaining

For immunostaining, colonies of undifferentiated hiPSCs and enzymatically-dispersed hiPSCs-CMs were fixed with 4%-paraformaldehyde, permeabilized with 1%-Triton-X-100 (Sigma), blocked with 5%-horse serum and 1% bovine serum albumin. Specimens were then incubated overnight at 4°C with primary antibodies targeting: Tra-1-60, Oct-4, connexin-43(Cx43), plakoglobin (PKG) and beta-catenin (all from Santa Cruz), Desmin (Thermo scientific), Nanog (Abcam), α-fetoprotein(AFP) (Cell Marque), Nestin and cardiac troponin I (cTnI) (Chemicon), SSEA-4 (R&D), sarcomeric α-actinin (Sigma), plakophilin-2 (PKP2) (Acris, Germany) . The preparations were incubated with secondary antibodies: donkey anti-mouse Alexa fluor 488-labeled immunoglobulin G (IgG) and Goat anti-mouse cy3-Immunoglobulin G
subclass2b (IgG2b), donkey anti-rabbit Alexa fluor® 488-labeled IgG, donkey anti-goat Cy2-labeled IgG and donkey anti-mouse Alexa -labeled IgM (all from The Jackson Immunoresearch Laboratories, West Grove, Pa) at a dilution of 1:200 for 1 hour. Nuclei were counterstained with DAPI (1:500, Sigma). The preparations were examined using a laser-scanning confocal microscope (Zeiss LSM-700). Alkaline-phosphatase staining of hiPSCs colonies was performed using Sigma detection kit.

The analysis of the immunofluorescence data were performed using images that were acquired using similar parameters and was conducted with the ImageJ software (NIH) and the image processing package Fiji1. Images were analyzed using pre-defined constant setting and a consistent automated densitometry algorithm. The density of the punctuate desmosomal (PKP2, Plakoglobin) and gap junction (Cx43) immunosignals was normalized to the total cardiomyocyte area (determined as the area positively stained for cTnI). For β-catenin nuclear density assessment, the ratio between nuclear β-catenin (co-localized with DAPI staining) and the total cellular β-Catenin signal in cTnI positive cells was calculated by the coloc2 analysis package in Fiji1.

**Gene expression analysis**

RNA was isolated using the RNeasy-plus micro-kit (Qiagen). Reverse transcription into cDNA was conducted with the high-capacity cDNA reverse-transcription kit (Applied-Biosystems). Real-time PCR studies were conducted in triplicates using the Fast-SYBR Green-Master-mix and Taq-Man® Fast Universal PCR Master Mix (Applied –Biosytems) for SYBR-Green and Taq-Man detection, respectively. Primers are detailed in supplementary Table 1. Samples were cycled using the Fast ABI-7500 sequence detector. Conditions were: 20s at 95°C followed by 40 cycles of 3s-95°C and 30s-60°C. Cycle-threshold was calculated using default settings for the
real-time sequence detection software (Applied-Biosystems). Relative quantification was calculated according to the \( \Delta\Delta CT \) method for quantitative real-time PCR (using an endogenous control gene).

**Microelectrode array (MEA) recordings**

To characterize the electrophysiological properties of the ARVC-hiPSCs-CMs, a multielectrode array (MEA) recording system (USB-MEA-256 system, Multichannel Systems, Reutlingen, Germany) was used. The contracting areas were micro-dissected and plated on fibronectin-coated MEA plates. The MEA system allows simultaneous recordings from 252 titanium nitride-coated gold electrodes (30 \( \mu \)m) at high spatial (100 \( \mu \)m) and temporal (15 kHz) resolutions. The local activation time at each electrode was determined as the timing of the maximal negative time derivative of the unipolar signal (dV/dt\( \text{min} \)). The local activation times were then used to generate color-coded activation maps using custom-made MATLAB-based software. The recorded electrograms were also used to determine the local field potential (FP) duration (FPD). This parameter was previously shown to correlate with the action potential duration and to reflect the local QT interval. The estimated FPD was defined as the time interval between the initial deflection of the FP to return of the measured FP to baseline FPD measurements were normalized (corrected FPD [cFPD]) to the beating rate of the contracting areas with the Fredericia correction formula (cFPD=FPD/[RR]^{1/3}). To assess for the excitability and conduction velocity of the cells the field potential rise time of the cell was assessed. Field potential rise time was measured by blinded automated algorithm assessing the FPD rise time between 10% and 90% of voltage rise (ClampFit 10.3 software, Molecular devices, Sunnyvale, CA).
TUNEL staining

For TUNEL staining ARVC and control contracting areas were dissociated using 0.1% trypsin and plated on bovine fibronectin (1%) coated slides (all from Biological Industries, Israel). To induce apoptosis cells were exposed to serum free media for 20 hours (DMEM F12, Life Technologies) prior to fixation. TUNEL staining was carried out according to manufacturer protocol (In Situ cell detection kit, TMR red, ROCHE). Preparations were examined using a laser-scanning confocal microscope (Zeiss LSM-700). Analysis of the immunofluorescence data were performed using images that were acquired using similar parameters and was conducted with the ImageJ software (NIH).

Transmission electron microscopy

Spontaneously beating areas within the EBs (40 days of differentiation) were microdissected and fixed with cold 3.5%-glutaraldehyde in 0.1M sodium-cacodylate buffer. Specimens were post-fixated with 1% osmium-tetroxide and dehydrated using 50-100% ethyl-alcohol. For visualization, epon blocks were cut into thin-sections (70nm) and stained with 1% uranyl-acetate followed by 0.4% lead-citrate. Transmission electron microscopy was carried out using JEM 1011 (Jeol, Tokyo, Japan). Images were acquired and analyzed using Gatan digital micrograph software (Warrendale, PA). Quantitative morphological measurements were conducted by an experienced operator blinded to the studied specimens.

References:

Legends for Supplementary Figures

Supplementary Figure 1: Clinical characteristics of the second ARVC patient.

[A] Electrocardiographic tracing showing the development of ventricular tachycardia in the second ARVC patient during programmed electrical stimulation. [B] Anteroposterior (AP) view of the electroanatomical unipolar voltage map of the RV of the second ARVC patient. Note the extensive area of low voltage (red indicates unipolar electrograms<4mV), corresponding to the diseased regions in the RV inflow, outflow, and apex. [C] Sequencing of the plakophilin-2 (PKP2) gene in hiPSCs derived from the second ARVC patient (ARVC-B, right-panel) and of the healthy-control hiPSCs (left-panel). Note in the affected allele in the ARVC-B hiPSCs the presence of a deletion of ACAG in positions 148-151 in exon 1 (c.148_151delACAG/N).

Supplementary Figure 2: Characterization of the hiPSCs derived from the second ARVC patient (ARVC-B).

[A] Phase-contrast of the generated ARVC-B hiPSCs colonies demonstrating typical embryonic stem cell colony morphology. [B] Positive staining of the ARVC-B hiPSCs colonies for alkaline-phosphatase. [C] Immunofluorescent staining of the ARVC-B hiPSCs colonies for the pluripotency markers OCT4, NANOG, SSEA4, and TRA-1-60. [D] Immunostaining of in-vitro differentiating EBs for Desmin (mesoderm), alpha-Fetoprotein (AFP, endoderm) and Nestin (ectoderm). Scale-bars for A-D: 100µm. [E] Real-time quantitative PCR showing the down-regulation of OCT3/4, KLF4 and SOX2 transgenes in the ARVC-B-hiPSCs. Values are normalized to the house-keeping gene RPL-7 and expressed as mean±SEM. Expression values (RQ(%)- Relative Quantitation (Percentage)) are relative to HEK293T cells (HEK) transiently transfected with the three plasmids to produce reprogramming virions. [F] Real-time quantitative
PCR evaluating the endogenous levels of the pluripotency genes *NANOG*, *FOXD3*, and *OCT 3/4* in healthy-control fibroblasts, ARVC-B fibroblasts and ARVC-B hiPSCs clones. Values are normalized to the house-keeping gene *RPL-7* and expressed as mean±SEM. Expression values (RQ) are relative to levels in the patient's fibroblasts.

Supplementary Figure 3: Desmosomal gene expression pattern and immunostainings

[A] Immunostaining of differentiating ARVC-B hiPSCs-CMs for the sarcomeric proteins cTnI (left) and α-actinin (right). [B] Real-time PCR analysis quantifying the expression of desmosomal proteins genes in the ARVC-B hiPSCs-CMs. Note the significant reduction in the expression of *PKP2* when compared to healthy-control cells (*p<0.05*). No significant differences were noted in the expression levels of *JUP* (Plakoglobin) and *DSP* (Desmoplakin). Values are normalized to the house-keeping gene *RPL-7* and expressed as mean±SEM. Expression values (RQ (%)) are relative to control hiPSC-CMs. [C-E] Immunostainings of the ARVC-B hiPSCs-CMs for the desmosomal and gap-junction proteins. Note the reduced immunosignal levels of *PKP2* (C), Plakoglobin (D) and Cx43 (E) in the ARVC-B hiPSCs-CMs (right-panel) when compared to healthy-control hiPSC-CMs (left-panel). Scale-bars for A,C-E: 25µm.

Supplementary Figure 4: Ultrastructural characterization of the ARVC-B hiPSCs-CMs.

Transmission electron microscopy image of the ARVC-B hiPSCs-CMs. [A-B] High-magnification images of distorted desmosomes (D) in ARVC-B hiPSCs-CMs. Note the presence of a structurally abnormal desmosome with a hazy configuration and a widened internal and
external desmosomal gap-widths. [C] Low-magnification image of the ARVC-B hiPSCs-CMs demonstrating the presence of lipid-droplet (L) clusters within the studied cardiomyocytes. 

[D] Low-magnification image of the ARVC-B hiPSCs-CMs showing distorted desmosomes and accumulation of intracellular lipid-droplet clusters in the same cell. Abbreviations: S-sarcomere, Nuc-nucleus, M-mitochondria, L-lipid droplets, D-desmosome.

Supplementary Figure 5: Apoptosis in ARVC and healthy-control hiPSCs-CMs
The rate of apoptotic cells was evaluated in cells dissociated from contracting areas of healthy-control and ARVC-hiPSCs. To induce apoptosis, cells were exposed to serum starvation for 20 hours. Quantitative analysis revealed a significantly higher proportion of TUNEL positive nuclei in ARVC cells (3.8±1%) when compared to control cells (1.0±0.6%, p=0.04).

Supplementary Figure 6: β-catenin nuclear density in ARVC and control cardiomyocytes
[A] Immunofluorescence staining for β-catenin in healthy-control (upper-panel) and ARVC (lower-panel) cardiomyocytes. The left-panel demonstrates the superposition of the nuclear staining (DAPI, blue), β-catenin staining (green) and the sarcomeric-α-actinin staining (red). Note the presence of nuclear β-catenin immunosignal in the healthy-control cells (arrows) but not in the ARVC cells. (Bar=50µm). (B) Quantitative assessment of the nuclear β-catenin density (a surrogate marker for canonical Wnt pathway activation). Note that the ARVC-hiPSCs-CMs displayed significantly reduced β-catenin density (5.2%±1.5%) when compared to healthy-control cells (12.1%±1.8%, p=0.02, n=7).
Supplementary Movie 1: Beating ARVC-derived hiPSC-CMs.

Supplementary Movie 2: Action-potential propagation within ARVC-hiPSCs-CMs as recorded by the multielectrode array (MEA) mapping system.
<table>
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Control

ARVC-B
c.148_151delACAG/N
p.T50SfsX110

Supp. Figure 1
Supp. Figure 2 - continue

E

![Bar chart showing expression levels of Tg OCT4, Tg SOX2, and Tg KLF-4 in Trans. HEK293 and ARVC-B hiPS cells.]

F

![Graphs showing expression levels of NANOG, FOXD3, and OCT-4 in Cont-Fib, ARVC-B Fib, and ARVC-B undif cells.]
Supp. Figure 3

A

B

![Image of cellular fluorescence with markers cTNI and sActinin](image)

![Bar chart showing expression levels of PKP2, JUP, and DSP in control and ARVC-B conditions](image)
C

Control

ARVC-B

D

cTNI PKP-2

E

cTNI Cx43

Plakoglobin

cTNI PKP-2

Plakoglobin

Control ARVC

−B
Supp. Figure 5

% Tunnel positive

\[ p = 0.04 \]
Supp. Figure 6

A

Control

ARVC

B

Beta catenin nuclear density (percentage)

Control

ARVC

*p = 0.02