Maturation-Based Model of Arrhythmogenic Right Ventricular Dysplasia Using Patient-Specific Induced Pluripotent Stem Cells

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Cellular reprogramming of somatic cells to patient-specific induced pluripotent stem cells (iPSCs) enables in-vitro modeling of human cardiac disorders for pathogenic and therapeutic investigations. However, using iPSC-derived cardiomyocytes (iPSC-CMs) to model an adult-onset heart disease remains challenging because of the uncertainty regarding the ability of relatively immature iPSC-CMs to fully recapitulate adult disease phenotypes. Arrhythmogenic right ventricular dysplasia (ARVD) is an inherited cardiomyopathy characterized by pathological fibrofatty infiltration and cardiomyocyte (CM) loss predominantly in the right ventricle (RV), leading to heart failure and lethal arrhythmias. Over 50% of affected individuals have desmosome gene mutations, most commonly in PKP2 encoding plakophilin-2. Using Yamanaka’s pluripotent factors, we generated iPSC lines from ARVD patients with PKP2 mutations. We first developed a method to induce metabolic maturation of iPSC-CMs and showed that induction of adult-like metabolic energetics from an embryonic/glycolytic state is essential to model an adult-onset cardiac disease using patient-specific iPSCs. Furthermore, we showed that coactivation of normal peroxisome proliferator-activated receptor (PPAR)-α and abnormal PPARγ pathways in ARVD iPSC-CMs resulted in exaggerated CM lipogenesis, CM apoptosis, Na+ channel downregulation and defective intracellular calcium handling, recapitulating the pathological signatures of ARVD. Using this model, we revealed novel pathogenic insights that metabolic derangement in an adult-like metabolic milieu underlies ARVD pathologies, enabling us to propose novel disease-modifying therapeutic strategies.

Key Words: Fatty acid oxidation; Induced pluripotent stem cell-derived cardiomyocytes; Metabolic maturation; Peroxisome proliferator-activated receptor-γ; Reactive oxygen species

Cardiovascular disease is a major health concern and cause of death worldwide.1 Recent advances in the cellular reprogramming of somatic cells from patients with heart diseases into induced pluripotent stem cells (iPSCs) have enabled the generation of cardiomyocytes (CMs) for myocardial repair4–8 and in-vitro modeling of human inherited cardiac disorders.9–20 However, CMs derived from human embryonic stem cells or iPSCs (hESC-CMs or iPSC-CMs) displayed heterogeneous and immature phenotypes. In fact, most published iPSC-based cardiomyocyte models used immature patient-specific iPSC-CMs and cultured them as single cells or a monolayer of cells in Petri dishes with stiff plastic surfaces. As a result, exaggerated CM pathologies and arrhythmia frequency appeared within 30 days in these non-physiological models, rendering their clinical relevance doubtful.19 The rapid-onset pathologies and exaggerated arrhythmias with these iPSC-based models in culture deviated greatly from the clinical course of the cardiac diseases. Thus, there is a tremendous need to develop methods of inducing maturation of primitive iPSC-CMs so that better and more clinically relevant cardiac disease models can be established for pathogenic and therapeutic investigations.

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) and Its Pathogenesis

ARVD/C (termed ARVD here for simplicity) is an inherited cardiomyopathy with a median age of onset at 26 years and most of the identified mutations in genes encoding cardiac desmosomes, which include plakoglobin (JUP), plakophilin-2 (PKP2), desmoplakin (DSP), desmoglein-2 and desmocollin.21–25 Pathologic hallmarks of ARVD are progressive fibro-fatty replacement of CMs with increased CM apoptosis primarily in the RV, leading to sudden death in the young (<35 years old).
tein (Pkg). This abnormal Pkg nuclear localization was suggested to compete with and decrease the binding of β-catenin to the TCF/LEF transcription factor complexes and led to low β-catenin activities and adipogenic transdifferentiation of CMs.

The same group of researchers also used genetic fate mapping techniques in mouse ARVD models to indicate that islet-1 positive (Isl1+) cardiac progenitor cells are the source of adipocytes in ARVD hearts. However, misuse of terminologies for adipogenesis and later conflicting data from other groups argue against the validity of this Pkg-β-catenin hypothesis. First, activation of peroxisome proliferator-activated receptor-γ (PPARγ) and its target genes was used as the marker for "adipogenesis (adipocyte formation)", which is absolutely incorrect and misleading. Second, PPARγ and its target genes are normally activated in several non-adipocyte tissues (eg, endothelial cells and macrophages), and could be abnormally activated by pathological conditions in liver (fatty livers) and CMs (diabetic cardiomyopathy). Therefore, activation of PPARγ and its target genes indicates the active process of "de novo lipogenesis" rather than cell transdifferentiation toward adipocytes. Also, the proper lineage marker for identifying adipogenic transdifferentiation of CMs (Pkg). This abnormal Pkg nuclear localization was suggested to compete with and decrease the binding of β-catenin to the TCF/LEF transcription factor complexes and led to low β-catenin activities and adipogenic transdifferentiation of CMs.

Clinical criteria to diagnose ARVD are well established, but the pathogenic processes of ARVD are difficult to study because: (1) obtaining cardiac samples from the early stages of human ARVD hearts is not possible because ARVD is commonly diagnosed at the advanced stage or postmortem, and (2) primary cardiac tissues from symptomatic ARVD patients are rarely available for mechanistic analysis because of safety concerns around taking large biopsy samples of cardiac tissues. These limiting factors impose significant constraints in developing therapies for human ARVD. Currently, no therapy is available to stop the progression of ARVD pathologies except inserting electronic cardiac defibrillators to prevent sudden cardiac death.

The pathogenic mechanisms by which desmosomal mutations cause CM loss, fibrofatty infiltration and lethal arrhythmia remain poorly understood. Experimental data from animal and cultured cell line models led to conflicting and conflicting results. Early experiments with DSP knockdown in murine HL-1 atrial tumor CMs and cardiac-specific DSP knockout (KO) mice suggested that DSP deficits resulted in strong oil-red staining in CMs and nuclear translocation of Plakoglobin proteins.
adipocytes is perilipin-1 (PLIN1), which is only expressed in adipocytes. Moreover, the majority of human ARVD heart tissue samples demonstrated significant downregulation of Pkg proteins without abnormal nuclear translocation and cardiac-specific KO of Pkg can reproduce ARVD pathologies in mouse hearts, indicating that loss of function in Pkg, rather than Pkg competition with β-catenin (gain of function), is responsible for eliciting ARVD pathologies.

Establishing iPSCs From Patients With Clinical ARVD and PKP2 Mutations

We have generated and fully characterized 2 sets of ARVD PKP2 mutant iPSC lines from 2 unrelated ARVD patients. The first ARVD patient has homozygous c.2484C>T mutations in PKP2 proteins (Pkp2) with frame-shifted C-terminals failing to anchor Pkg to the sarcolemmal membrane (JK lines). The second patient has a heterozygous c.2013delC mutation in PKP2 (termed delC PKP2 mutation) that causes frame-shift and premature termination in exon 10, leading to degradation of mutant PKP2 mRNAs and a PKP2 haploinsufficiency phenotype. Importantly, we observed abnormal nuclear translocation of Pkg (only in CMs contacting plastic culture surfaces, unpublished observations) and very low β-catenin activity in both PKP2 mutant ARVD iPSC-CMs at baseline but no exaggerated lipogenesis or apoptosis in ARVD iPSC-CMs was found after culturing for 2–3 months, indicating that Pkg nuclear translocation and low β-catenin activities are insufficient to produce ARVD pathologies. Moreover, we did not observe any CM transdifferentiation to adipocytes in our cardiac progenitor cell populations derived from ARVD iPSCs under any conditions. These results are consistent with a loss of function role of Pkg in the pathogenesis of ARVD hearts.

Designing the Method of Metabolic Maturation Induction

The major metabolic differences between embryonic and adult
CMs are: (1) embryonic CMs use mainly glycolysis and lactate for energy production, and (2) adult CMs produce most energy via fatty acid oxidation (FAO) but retain capability to readily switch to glucose or other substrate utilization when fatty acid is not available or FAO is compromised. At baseline, the potential energy substrates in our culture media (DMEM) are 25 mmol/L D-glucose, 1 mmol/L pyruvate, 2 mmol/L glutamate, essential amino acids, 1 mmol/L NEAA, lipids from de novo lipogenesis and minimal energy substrates contained in the 2% fetal bovine serum. This culture medium with high glucose content is very standard for growing human ESC/iPSC-CMs.

We then used insulin, a glucocorticoid (dexamethasone (Dexa)) and 3-isobutyl-1-methyl-xanthine (IBMX, a phosphodiesterase inhibitor) to develop a 3-factor (3F) protocol to drive metabolic maturation (Figure 1). Among these 3 factors, glucocorticoid is catabolic and can increase mitochondrial oxidation of glucose and fatty acids in muscles but promote both lipogenesis and lipolysis (triacylglycerol turnover) in adipocytes. Insulin is known to inhibit FAO, but promote glucose utilization, endogenous de novo lipogenesis and lipid storage in various cell types. Also, based on our metabolic assays, both normal and ARVD iPSC-CMs at baseline showed a low oxygen consumption rate (OCR) from FAO but high levels of glycolysis by extracellular acidification rate (ECAR) measurement, indicating that glycolysis is the main mechanism of energy production for immature iPSC-CMs in culture.

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The number in each column represents the number of biological replicates tested. *P<0.05; NS, no significant difference by ANOVA. P values are shown when unpaired t-test was performed. ARVD, arrhythmogenic right ventricular dysplasia; FAO, fatty acid oxidation; iPSC-CM, induced pluripotent stem cell-derived cardiomyocyte; NAC, N-acetyl-cysteine; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TCA, tricarboxylic acid or Krebs cycle. Adapted from Kim C, et al.16

**Figure 3.** Functional assays of glucose utilization vs. FAO in PKP2 mutant iPSC-CMs after various treatments. (A) Simple diagram illustrating substrate utilization pathways. Using a Seahorse XF96 Extracellular Flux Analyzer, we determined the degree of FAO by measuring the etomoxir (100 µmol/L ETO, a specific CPT-1 inhibitor) -blocked components of OCR and glycolysis by measuring 2-deoxyglucose (50 mmol/L 2-DG) blocked components of ECAR (see detailed methods). Real-time measurement of (B) OCR and (C) ECAR after ETO showed a rapid ~21% compensatory increase in glycolysis only in the 3 factor (3F) condition (a switch in energy substrates). In contrast, after 5 factor (5F), ETO transiently decreased ECAR by ~45% (pyruvate oxidation, green arrow) and followed by ~28% increase in ECAR (compensatory increase in glycolysis). (D) FAO (blue area) increased greatly with a small increase in (E) glycolysis (blue region) by 3F metabolic induction. In contrast, 5F induced a pathological burnt-out state with larger decrease in FAO (red in D) than the decrease in glycolysis (red in E). Combining the data shown in A-E, the results support that both normal and mutant iPSC-CMs display embryonic metabolism at the baseline, and show significantly increased FAO after 3F with an ability to switch between FAO and glucose utilization (an adult-like metabolic pattern). ARVD iPSC-CMs after 5F behave like failing cardiomyocytes with pathological glucose-dominant metabolism. The number in each column represents the number of biological replicates tested. *P<0.05; NS, no significant difference by ANOVA. P values are shown when unpaired t-test was performed. ARVD, arrhythmogenic right ventricular dysplasia; FAO, fatty acid oxidation; iPSC-CM, induced pluripotent stem cell-derived cardiomyocyte; NAC, N-acetyl-cysteine; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TCA, tricarboxylic acid or Krebs cycle. Adapted from Kim C, et al.16
in cardiac muscles. Increased fatty acid synthesis would produce distinct phosphatidylcholine species that can act as the endogenous ligand to specifically activate PPARα in the liver or in CMs. For these reasons, we designed the 3F protocol to activate PPARα and increase lipogenesis and FAO in iPSC-CMs. Our functional assay by the Seahorse analyzer without added external fats demonstrated that both ARVD and normal iPSC-CMs after 3F treatment had higher absolute OCRs from both fatty acid and glucose oxidation than unstimulated iPSC-CMs at 60 days after differentiation, supporting that our 3F protocol indeed induced mitochondrial maturation with active oxidative phosphorylation. No significant apoptosis was found in the normal and ARVD iPSC-CMs after 3F induction. Thus, activation of PPARα alone by 3F in iPSC-CMs induces an adult-like metabolism without provoking any ARVD pathology (Figure 2).

**Metabolic Maturation-Based Pathogenic Induction of ARVD Pathologies**

PPARα is the major transcriptional regulator of fatty acid metabolism in adult CMs. In contrast, PPARγ should be minimally activated in normal CMs. However, the PPARγ pathway has been reported as abnormally hyperactivated in RV tissue samples of ARVD hearts, and transgene-induced overexpression of PPARγ in mouse CMs could lead to dilated cardiomyopathy. Based on these lines of evidence, we added 2 PPARγ activators, 5µM rosiglitazone and 200µM indomethacin, to the 3F protocol to further induce abnormal PPARγ overactivation in beating embryoid bodies (EBs) formed by the 5-factor (5F) protocol (Figure 2A). We found that ARVD iPSC-CMs manifested ARVD pathologies only after inducing adult-like metabolism and abnormal activation of the PPARγ pathway. Coactivation of the normal PPARα and abnormal PPARγ pathways in ARVD iPSC-CMs resulted in exaggerated lipogenesis in CMs, CM apoptosis, Na+ channel downregulation, and defective intracellular calcium (Ca2+ ) handling capability, recapitulating the pathological signatures of ARVD (Figures 2B–D). We further showed that the endogenous PPARγ activator 13-hydroxyocta-decadienoic acid (13-HODE, a major component of the oxidized low-density lipoprotein) could replace rosiglitazone and indomethacin in the 5F protocol for abnormal PPARγ activation, leading to extensive lipogenesis and apoptosis in mutant iPSC-CMs (Figures 2E–F). Importantly, overexpression of wild-type PKP2 in ARVD iPSC-CMs reversed all ARVD pathologies, supporting that observed pathological phenotypes are the result of mutated PKP2 genes in iPSCs.

**Potential Therapeutic Strategies for Treating ARVD Pathologies**

We first showed that PPARγ antagonists (e.g., GW9662 (GW) or T0070907 (T007)) rescued all ARVD pathologies (Figures 2E–F) and reactive oxygen species (ROS) scavengers [N-acetyl-cysteine (NAC) or ascorbic acid (AA)] curtailed CM apoptosis in our ARVD in-vitro model (Figure 2G). Of note, activation of PPARγ alone, or blockade of PPARα-dependent metabolism alone with an antagonist, GW6471, after 5F treatment did not induce significant ARVD pathologies, supporting the crucial role of PPARα/PPARγ coactivation in mediating ARVD pathogenesis. Moreover, the absolute levels of FAO in ARVD iPSC-CMs are always 1.5–2-fold higher than those of normal iPSC-CMs after 3F metabolic maturation induction (see figure 4H). This higher FAO flux in ARVD iPSC-CMs might explain why some ARVD patients are excellent athletes when young before the pathological processes kick in. However, we hypothesize that this high FAO flux might also be responsible for the excessive ROS production in ARVD iPSC-CMs after PPARα/PPARγ coactivation but before ARVD iPSC-CMs reach the failing burnt-out state (see more below). Therefore, we dialed down FAO (using etomoxir (ETO) or β-oxidation [using 4 bromocrotonic acid (4 BCA)]) with an EC50 dose of the respective inhibitors, which reduced CM apoptosis (Figure 2H), indicating that high FAO flux is required for ARVD pathologies.

**Distinct Metabolic Phenotypes of Immature, Mature and Pathological ARVD iPSC-CMs**

Using the Seahorse XF96 Extracellular Flux Analyzer, functional assays of FAO and glycolysis in live cells revealed that normal and ARVD iPSC-CMs had dominant glycolytic energetics (an embryonic pattern) at baseline (Figures 3A–E). After activation of PPARα by 3F, mutant and normal iPSC-CMs displayed slightly higher levels of glycolysis but significant activation of FAO (an adult-like pattern) when compared with the un-induced, baseline conditions (zero factor). Compared with 3F induction, mutant PKP2 iPSC-CMs after 4 weeks of pathogenic coactivation of PPARα and PPARγ by 5F demonstrated overall depressed energy metabolism, with more FAO reduction than 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. Thus, results from immunocytotoxic, genetic, and metabolic assays strongly support that coactivation of PPARα and PPARγ by hormones and small molecules accelerates the pathogenesis and establishes an efficient in-vitro model of ARVD, recapitulating the metabolic and pathological signatures of failing ARVD hearts within 2 months.

**Concluding Remarks and Future Perspective**

Using patient-specific mutant PKP2 iPSC-CMs grown in a 3D beating EB format and induced by various pathogenic conditions, we accelerated the pathogenesis of an adult-onset disease. We demonstrated the importance of PPARα-dependent metabolic maturation, PPARγ coactivation, ROS production and FAO in the pathogenesis of ARVD. This efficient in-vitro iPSC-CM-based model recapitulates the pathognomonic features of the ARVD heart and enables pathogenic investigation and therapeutic screens. Future investigations regarding how mutant Pkp2 leads to abnormal PPARγ activation in ARVD CMs will further deepen our understanding of this unfortunate disease and pave the way for developing novel therapeutic strategies.

Furthermore, we observed only abnormal “lipogenesis” in CMs but not exaggerated adipogenesis (adipocyte formation) in our CM-centric model, suggesting that non-CMs must be involved in the abnormal adipogenesis in ARVD hearts. Importantly, we did not observe any transdifferentiation of iPSC-CMs to adipocytes (perilipin-1 positive cells). Of note, cardiac fibrosis is also a main finding in ARVD hearts. Because human hearts contain significant numbers of non-CMs elucidating the subtype(s) of non-CMs that account for the abnormal adipogenesis and/or fibrosis will further improve our in-vitro model and render it more clinically relevant. Only with clinically relevant models, therapeutic screens can then be conducted to develop feasible therapies for human ARVD. Furthermore, the metabolic maturation induction method can be improved further with the addition of several postnatal hormones to achieve true adult levels of metabolic maturation. Patterning
of iPSC-CMs on hydrogels with cardiac stiffness might further improve the relevance of these iPSC-CM based cardiac disease models in vitro. Additional abnormalities reported in ARVD hearts or models, such as Kir2.1 channels (mediating Ik1 currents), connexin 43 (Cx43), Ca2+-handling capabilities, and synapse-associated protein-97 trafficking proteins should be also investigated with our in-vitro models to elucidate the respective pathogenic mechanisms for each pathology and to develop novel therapies.

Finally, patient-specific iPSC-CMs have been shown to be useful in studying the pathogenesis of inherited cardiac disorders. Many so-called cardiac disease models have been established using immature and isolated patient-specific iPSC-CMs. As a result, rapid-onset pathologies (within 30 days) and spontaneous arrhythmias in these iPSC-CM based models are frequently observed in these non-physiological milieu and without any maturation or pathogenic induction. These spontaneous and exaggerated pathologies deviate greatly from the clinical course of the postnatal cardiac disorders, which usually take years to manifest their clinical phenotypes. The clinical relevance of these iPSC-CM based cardiac disease models is therefore questionable. Thus, we must devote effort and time into studying the mechanisms of CM maturation so as to build better and more clinically relevant in-vitro models before an applicable therapeutic screen can be pursued.

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Disclosures

The authors report no conflicts.

References

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