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Induced Pluripotent Stem Cell-Derived Cardiomyocytes: A Platform for Testing For Drug Cardiotoxicity

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Abstract

Off-target cardiotoxicity has been a significant impediment to the development of new drugs. Traditional platforms for screening for cardiotoxicity are both overly sensitive and limited in their ability to predict cardiotoxicity that is often only uncovered after years of clinical use. A major impediment has been the lack of a human cardiomyocyte cell line. The recent discovery that adult somatic human cells (white blood cells or skin fibroblasts) can be reprogrammed into pluripotent stem cells (hiPSCs) and then differentiated into beating cardiomyocytes (hiPSC-CMs) provides an exciting new platform for drug cardiotoxicity and efficacy testing. One major advantage of using patient-derived hiPSC-CMs for drug testing is their ability to recapitulate population genetic variations (single nucleotide polymorphisms) that influence drug toxicity, providing a powerful new tool in the field of pharmacogenomics and personalized medicine.

Keywords

Cardiotoxicity; human induced pluripotent stem cells; cardiomyocyte; pharmacogenomics; cell signaling; chemotherapeutics

Introduction

When developing new drugs, testing for cardiotoxicity is one of the key components of early toxicity screening. All new drug candidates are currently screened for effects on the voltage-gated potassium channel that lead to prolongation of the QT interval. Up to 30% of potential new drugs have to be abandoned because they fail these preliminary tests, even though the false positive rate may be as high as 20–25% (1), and others may advance to clinical trials, as the false negative rate is equally high. Traditionally, these preliminary screening studies have been performed in non-cardiac cells made to artificially overexpress the particular channel of interest, and translation of these results to the human heart can be complicated by species differences and the lack of many normal cardiomyocyte cell signaling components in these cell systems (2). Furthermore, these platforms cannot recapitulate the effect of

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common human gene variants, known as single nucleotide polymorphisms (SNPs) on drug efficacy and toxicity. The lack of a human cardiomyocyte cell line has been a major impediment to further advances in this field.

Over the past decade, advances in stem cell technology have now provided researchers with the ability to routinely generate human cardiomyocyte cell lines from induced pluripotent stem cells (hiPSCs-CMs), providing a powerful new platform to test drugs for both toxicity and therapeutic benefit. hiPSC-CMs offer the potential to develop a better understanding of how patient-specific reactions to a drug are driven by interactions between the patient's genome and epigenetic and environmental factors (3). However, as useful as hiPSC-CMs have been for the evaluation of channelopathies such as long QT syndrome, there are many important immaturities in these cells that may make them less than ideal for studies on drug effect/toxicity on contractile function.

Cardiotoxicity of Common Drugs

Over one million adverse drug reactions (ADEs) are reported in post-market surveillance to the Food and Drug Administration (FDA) each year (4). Adverse reactions specific to the heart range from minor effects on heart rate and blood pressure that have little clinical significance, to life-threatening arrhythmias (e.g. drugs that prolong the QT interval), valve pathology (e.g. the diet aid FenFen) and cardiomyopathy (the anti-cancer anthracyclines). Between 1953 and 2013, 63 drugs have been withdrawn from the market because of serious cardiovascular adverse events. Because some of these cardiotoxicities may be relatively rare, they may not be discovered during the initial phase II or III clinical trials, and several drugs have been withdrawn from the market only many decades after initial FDA approval. Benfluorex (used for weight reduction) was withdrawn only 33 years after its initial introduction (5). Children may be at particular risk for cardiotoxicity, in part due to the longer time-at-risk after drug exposure and in part due to the potential for increased susceptibility of the immature heart to injury. The chemotherapeutic drugs are of particular concern, with reported cardiotoxicity ranging from 8 to 25% for anthracyclines like doxorubicin, 2–35% for the tyrosine kinase inhibitors, 7–28% for the HER2 monoclonal antibody trastuzumab, and 5–30% for the microtubule stabilizer paclitaxel (3).

Pharmacogenomics and Advances in Personalized Medicine

One of the greatest potential uses of hiPSC-derived cardiomyocytes (hiPSC-CMs) is in the rapidly growing field of pharmacogenomics. In the past, if a patient experienced a toxic reaction to a drug, it was regarded simply as an undesirable side-effect of that drug. We now recognize that many adverse reactions are related to the complex interaction of a specific drug with a specific patient's genetic makeup that predisposes them to having a toxic reaction. These genetic differences may be mediated through differences in drug absorption, distribution, metabolism or excretion (ADME) and are the subject of considerable research. One of the tools used to elucidate these genetic effects is the genome-wide association study (GWAS), which analyzes large patient populations to identify single gene polymorphisms (SNPs) that influence treatment outcomes. For example, one GWAS study of over 100,000 patients identified 68 SNPs that were associated with length of the QT interval (6). However,

sometimes, gene variants initially identified as predictive of clinical outcomes are found on further study to not have the effect that was initially predicted. One example is the Ile164 variant in the β 2-adrenergic receptor, which was initially thought to be associated with worse outcomes in patients after an initial diagnosis of heart failure (7). Subsequent larger studies failed to confirm this association. Another example is the effect of the Gly389 variant on the clinical response to β -blocker therapy (8). The effect of this SNP was initially demonstrated for the β -blocker bucindolol, and would have been clinically very important if it had been shown to be a class effect (i.e. true for all β -blockers), but subsequent studies have shown that this effect is bucindolol-specific, and does not alter the efficacy of other β -blockers such as carvedilol or metoprolol (9).

When a GWAS variant thought to be predictive of a pharmacologic effect is first discovered, its effect is reported by an odds ratio (representing the increased risk of the gene variant causing a particular effect) and a statistical significance (p) value. Many GWAS studies for common diseases (e.g. Type II diabetes, coronary artery disease) have found gene variants of high statistical significance, but with low odds ratios (1.3–1.5), suggesting that the influence of that specific SNP is real but relatively small. Even for GWAS findings of higher odds ratios, these population-based studies provide only the first step towards application to clinical practice. Before widespread adoption in clinical testing, the effect of each gene variant must be validated using either *in vitro* or *in vivo* models. Unfortunately, rodent models may not accurately recapitulate the cardiotoxicity of drugs in humans; the tyrosine kinases inhibitors (used as chemotherapeutics) are a great example (10). This is where the availability of a human cardiomyocyte cell line has the potential to revolutionize this field. If these GWAS studies can be confirmed, then drug therapy could be tailored to individual patients based on their genome, with the result being decreased toxicity and increased clinical effectiveness.

Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs)

Attempts to culture human cardiomyocytes have been limited due to the extreme difficulty in propagating these cells *in vitro*. Thus, it was a major advance when methods were developed to generate induced pluripotent stem cells (iPSCs) directly from patient-derived somatic cells (skin fibroblasts, white blood cells, adipocytes) (11). First achieved in mouse cells in 2006 and then in human cells in 2007, this breakthrough mostly obviated the need for using human embryonic stem cells, and was recognized by award of the Nobel Prize to Drs. Shinya Yamanaka and Sir John Gurdon in 2012.

Once iPSCs are derived from a patient's cells, they can then be differentiated into cardiomyocytes (hiPSC-CMs) using various protocols, most taking advantage of our knowledge of the molecular basis of cardiomyocyte differentiation from studies of cardiac developmental biology. The very initial studies generated spherical aggregates of cells known as embryoid bodies (EBs), in which only a small portion of cells would be actively contracting (12). Subsequently, methods were developed to differentiate monolayers of cells (13) and over the years, each subsequent advance dramatically increased the efficiency of

differentiation, from initially <10% to now >90% (14; 15). Further advances have been made by using chemically defined media that eliminates all animal-derived products (e.g. serum and bovine albumin) from the media, thus generating hiPSC-CMs in total xeno-free conditions (16). Further efforts have focused on scaling the production of hiPSC-CMs for higher throughput and in developing microtissues using combinations of hiPSC-CMs and other cardiac cells (e.g. fibroblasts, endothelial cells).

Current cardiomyocyte differentiation protocols result in the generation of mixed populations of cardiomyocytes, consisting of cells that electrically resemble atrial, nodal or ventricular cardiomyocytes. This mix of cell populations is less than ideal for studying the effect of drugs that predominantly affect ventricular structure and function. Furthermore, this cellular heterogeneity could adversely skew results in studies trying to model either atrial or ventricular arrhythmias. To overcome this limitation, one approach has been to use fluorescent reporters linked to atrial-specific (sarcolipin (17)) or ventricular (myosin light chain 2V (18))-specific genes and then using fluorescent cell sorting (FACS) to isolate populations of cells with higher purity.

Do hiPSC-CMs Sufficiently Recapitulate the Biology of Mature Cardiomyocytes?

The current generation of hiPSC-derived cardiomyocytes are developmentally highly immature, resembling embryonic or fetal rather than adult cardiomyocytes (19). Since they express most of the ion channels responsible for generation of the action potential (with the exception of KCNJ2 (I_{K1}) (20)), they have been used to successfully study the effect of many drugs on arrhythmia risk (see below) (21). In contrast, their sarcomeres are poorly aligned, and many components of cell signaling and of the critical process of calcium-induced-calcium release are absent or poorly expressed. These features include absent or lower expression of the sarcoplasmic reticulum (SR) components SERCA, RyR, calsequestrin (for Ca²⁺ buffering) and phospholamban and absence or severe immaturity of T-tubules, structures which are critical to the functioning of the mature cardiomyocyte, localizing Ca²⁺ release close to the myofilaments. Many key proteins are expressed as their fetal isoforms, e.g. troponin I *TNNI1* (22), which may have functional properties different from their mature (*TNNI3*) isoforms.

We and others have shown that hiPSC-CMs undergo considerable maturation just by extending their length of time in cell culture from the typical 20–30 days to 60–90 days (23; 24). For example, at 30d after differentiation hiPSC-CMs express predominantly β 2-adrenergic receptors, with significant expression of β 1-receptors (the dominant subtype in the adult heart) becoming apparent only after 60–90 days in culture (23). Critical to studies of drug cardiotoxicity, the signalling pathways that connect sympathetic stimulation to cardiac remodelling pathways (e.g. CaMKII) are poorly developed until at least 90 days in culture. Even when mRNA or protein of a specific signaling molecule was present, functionality of that molecule (e.g. phosphorylation as means of activating downstream effectors) was often not present until at least 90 days. Thus, if one were studying a drug that had its toxic effect through CaMKII activation, this effect would be absent in hiPSC-CMs at

earlier time points, and thus false negative results would be obtained. Maintaining hiPSC-CMs in culture for prolonged periods has many disadvantages, but fortunately, this process can be accelerated dramatically using bioengineered culture platforms that pattern the morphology of cardiomyocytes to develop length:width aspects (7:1) that more closely resemble those of adult cardiomyocytes, and also when grown on substrates that approximate the stiffness (10 kPa) of the normal heart (25). Engineered heart tissues (EHT), consisting of both cardiomyocytes and fibroblasts, also result in acceleration of hiPSC-CM maturation (26).

Another key challenge in this area has been in recapitulating the metabolic requirements of mature cardiomyocytes. Fetal cardiomyocytes utilize glucose for energy production via anaerobic glycolysis, in contrast to the mature heart which utilizes fatty acids via beta oxidation (27; 28). Immature hiPSC-CMs utilizing glucose could result in erroneous results when studying a drug which has its mechanism of action through alterations in fatty acid metabolism or mitochondrial function.

Examples of Using hiPSC-CMs for Assessment of Drug-Induced Cardiotoxicity

1. Arrhythmias

One of the most common drug cardiotoxicities is the propensity to induce life-threatening arrhythmias. As mentioned above, this is one feature that is routinely tested for all new drugs early in their process of development, and one that has blocked the development of many drugs both in pre-clinical *in vitro* tests or in clinical trials. Although subtle alterations in heart rate are quite common with many drugs (e.g. ADHD medications), this more serious effect manifests as prolongation of the QT interval on the ECG, and the risk for developing the ventricular arrhythmia torsades de pointes. At the cellular level, this is most often due to prolongation of phase III of the action potential, induced by altered activity of the delayed rectifier potassium current, I_{Kr} , encoded by the *KCNH2* gene (formerly known as *hERG*) (29). Drug effects on other ion channels regulating potassium, sodium or calcium currents can also result in QT prolongation or other deleterious effects on the action potential. Several studies in hiPSC-CMs have demonstrated that they accurately recapitulate the abnormal electrophysiology of the patients from whom they were derived: long QT syndrome (LQTS) (30; 31), LEOPARD syndrome (32) and arrhythmogenic right ventricular cardiomyopathy (ARVC) (33). The electrophysiologic effects in these cells can be measured using standard, but laborious, patch clamp techniques, or by using the more high throughput multiple electrode array (MEA). In one landmark study, Yazawa et al. used hiPSC-CMs from patients with Timothy syndrome to uncover previously unknown mechanisms for QT prolongation and to identify potential new drug therapies (34).

2. Contractile Dysfunction

Drugs that alter cardiomyocyte contractility may affect systolic function (inotropy), diastolic function (lusitropy) or both. In hiPSC-CMs these effects may also be manifested as alterations in cellular structure, e.g. sarcomeric disorganization, although sarcomere organization in hiPSC-CMs is far from normal in the first place. The anthracyclines, such as

doxorubicin, are one of the principal drugs that affect cardiomyocyte function. First developed in the early-1960s, these chemotherapeutic drugs are highly effective in treating many different malignancies, and are currently utilized in treating ~70% of childhood tumors, and ~50% of breast tumors. It is estimated that nearly 1 million individuals are exposed to anthracyclines each year in the U.S. Their use is limited, however, by a dose-related cardiotoxicity, and therefore the total dose utilized is usually limited, potentially reducing anti-tumor effectiveness. The incidence of significant cardiotoxicity has been reported as between 8 and 15%, however, more subtle cardiac effects such as elevated wall stress have been found in up to 65% of children, even at doses as low as 225 mg/m² (35; 36). The mechanisms of anthracycline-induced cardiotoxicity (largely generation of reactive oxygen species [ROS] and effects on mitochondria) are mostly different from those mediating its effects in tumor cells (mostly effects on DNA replication and repair, a critical effect in rapidly replicating cancer cells [Fig. 1]).

To examine whether a patient's genetic composition affects their likelihood of developing anthracycline-induced cardiotoxicity, Aminkeng et al. performed a GWAS study on 275 children enrolled in the Canadian Pharmacogenomic Network for Drug Safety, and discovered a gene variant (SNP) in RARG, the gene encoding retinoic acid receptor gamma, that was associated with an increased risk of cardiotoxicity (odds ratio 4.58) (37). This was confirmed in two replication cohorts, one from the Netherlands and the other from Stanford in the U.S. However, although RARG is known to play a major role in cardiac looping morphogenesis, its role in the mature heart was largely unknown. Thus, a mechanism to explain this GWAS finding was lacking. In addition to RARG, several other gene variants have been identified as potentially linked to the risk of anthracycline cardiotoxicity, some increasing and others decreasing that risk, although none have been confirmed in large patient cohorts (Table 1). This is a perfect example where the hiPSC-CM platform can have its greatest utility.

Burridge et al. demonstrated that hiPSC-CMs can be used to recapitulate the propensity for cardiotoxicity (38). hiPSCs were derived from two groups of women who had received anthracyclines for treatment of breast cancer: those who had developed cardiotoxicity (DOXTOX group) and those who had not (DOX group). When differentiated into hiPSC-CMs, and then exposed to doxorubicin, cells from patients who had developed cardiotoxicity showed increased susceptibility to doxorubicin injury *in vitro*: increased cell death, lower mitochondrial membrane potential, altered calcium transients and increase ROS generation (Fig. 2). RNA sequencing showed dramatic differences in gene expression after drug exposure between the two groups. These data suggested that hiPSC-CMs are a valid platform for studying anthracycline cardiotoxicity and could thus be used to study whether the GWAS-identified variants mentioned above were true risk-modifiers. Preliminary data from a collaboration between our group and Dr. Burridge (now at Northwestern University) have shown that hiPSC-CMs from patients with the RARG variant do have increased susceptibility to doxorubicin. The advantage of using the hiPSC-CM platform is that a comprehensive assessment of mechanism is possible: using these cells, RARG can be knocked out using CRISPR/Cas9 or other gene editing technology (which would be expected to further increase cardiotoxicity) or overexpressed (which would be expected to reduce cardiotoxicity). Control hiPSCs can be gene edited using CRISPR/Cas9 to introduce

the deleterious gene variant, which should then recapitulate the enhanced cardiotoxicity seen in the patient-derived cells. Similarly, hiPSCs from patients carrying the deleterious variant can be gene edited to correct the SNP, which should then rescue the enhanced cardiotoxicity. Finally, a panel of patient-derived hiPSC-CMs from patients with and without each SNP can be used to test new drugs for preventing or attenuating anthracycline cardiotoxicity.

Conclusions

Cardiotoxicity is a side effect associated with many drugs used to treat both cardiovascular and non-cardiovascular diseases. Screening for cardiotoxicity, especially prolongation of the QT interval, has eliminated many drugs early in the development pipeline, most appropriately, but the false positive and false negative rates are still unacceptably high. The development of a human cardiomyocyte-based platform for drug screening is being actively pursued in many arenas, and would represent a major advance in drug safety and efficacy testing.

Advances in pharmacogenomics have shown that an individual patient's gene polymorphisms can contribute to their susceptibility to the cardiotoxic effects of many drugs. GWAS studies are commonly used to identify these gene variants, however, once identified these gene variants must be confirmed and mechanisms for their effect defined. hiPSC-CMs represent a platform which has great potential for increasing the accuracy of these assessments, as drug responses in human cells are the best predictor of drug responses in the human heart. The generation of large biobanks of patient-derived hiPSC-CMs, representing patients with different genomic backgrounds, as well as from different racial and ethnic backgrounds, will allow us to more accurately identify drug efficacy and toxicity at the earliest stages of drug development.

Acknowledgments

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Highlights

- Off-target cardiotoxicity is found in up to 30% of new drugs and is a major limitation to drug development.
- Current methods for assessment of drug cardiotoxicity have unacceptably high false positive and false negative rates.
- Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) represent an exciting new platform for testing for drug efficacy and cardiotoxicity.
- hiPSC-CMs have the capacity to replicate the genetic diversity present in human drug responses.
- hiPSC-CMs open a new window in pharmacogenomics and personalized medicine

TUMOR

- Intercalates with DNA
- Interferes with DNA and RNA polymerase
- Inhibits topoisomerases (Top2 α specificity)

CARDIAC

- Generates reactive oxygen species (H_2O_2 , $^{\cdot}OH$)
- Peroxidation of membrane lipids and mtDNA
- Mitochondrial dysfunction
- Activation of apoptotic pathways
- Top2 β specificity

Figure 1.

The potential for developing biased protection against anthracycline cardiotoxicity: Different mechanisms by which anthracyclines affect tumor cells vs. cardiac cells.

Figure 2. hiPSC-CMs obtained from patients who had suffered from doxorubicin cardiotoxicity (DOXTOX) showed increased cell death on exposure to doxorubicin *in vitro* compare to hiPSC-CMs obtained from patients who had received doxorubicin but did not experience cardiotoxicity. From Burrige et al. (38).

Table I

Gene variants that have been associated with anthracycline cardiotoxicity in preliminary clinical studies.

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- **RARG:** Aminkeng et al. Nature Genetics 2015 (37)
 - **SLC28A3:** Visscher et al. J. Clin. Oncol. 2012 (39)
 - **UGT1A6:** Visscher et al. J. Clin. Oncol. 2012 (39)
 - **Carbonyl reductase 3 (CBR3):** Blanco et al. J Clin Oncol. 2012 (40)
 - **NAD(P)H oxidase complex (CYBA, NCF4, and RAC2):** Wojnowski et al. Circulation. 2005 (41)
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