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Stem Cell-Derived Models in Toxicology

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Cover Illustration: Confocal microscopy image of a spheroid microtissue of hiPSC-derived cardiomyocytes (Cellular Dynamics International, Madison, USA) and human umbilical vein endothelial cells (Zen-Bio Inc., Durham, USA) produced using the GravityTrap hanging drop method (InSphero AG, Schlieren, Switzerland), and immunostained for von Willebrand factor (green), sarcomeric protein myomesin (red), and DNA (blue); from Chapter 11, Zuppinger. Overlaid is a field potential transient signal recorded from spontaneously beating hSC-derived cardiomyocytes on the multielectrode array (MEA) platform (Axion BioSystems Inc., Atlanta, USA); from Preface, Clements.

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Preface

Unexpected adverse toxicity findings in drug development or postlaunch have resulted in numerous costly late-stage drug development failures and market withdrawals. To mitigate this risk, the pharmaceutical industry has adopted a range of assays to probe the liability of compounds earlier in the drug development process consistent with the mantra "fail faster, fail cheaper". In the drug discovery process, lead optimization is the ideal phase to screen for safety issues, since at this point failure is relatively cheap and the number of potential compounds for selection (~1000) is greatest. The currently available standard testing models include primary tissue and immortalized cell lines. While immortalized cell lines lend themselves to screening applications (e.g. relatively inexpensive, abundant, easy to handle), the assay end point can be overly simplistic, leading to false positives (i.e. compounds flagged as toxic which are in fact safe) and false negatives (i.e. compounds misidentified as safe when actually toxic). Primary tissue (typically rodent) is often useful for small investigative studies but is not applicable for screening applications, due to both cost and ethical concerns with regard to animal consumption (3Rs). Concerns also persist with the translation of this data to humans due to possible species differences. Consequently, there is increasing demand for more relevant and predictive nonclinical models for in vitro toxicity testing (see Chap. 1, Gintant and Braam).

Since the first descriptions of the differentiation of cardiomyocytes from human embryonic stem cells (hESC) more than a decade ago, there has been much speculation over the utility of these cells for drug safety assessment [1]. Human ESC and human-induced pluripotent stem cell-derived (hiPSC) models, together termed human stem cell-derived (hSC) models, offer an opportunity to provide a more predictive, integrated human model system that is amenable to high-throughput screening in preclinical drug safety assessment. The relative immaturity of these stem cell-derived models is well documented (e.g. [2]), which has led some to question the applicability of hSC-derived models in toxicological studies (i.e. "they are not old enough for drugs").

Although methods to produce hSC-derived models with more "adultlike" phenotypes are currently the focus of intensive research efforts, there is considerable data in the literature to suggest a role for the current iteration of this technology. Accordingly, the potential application of hSC-cardiomyocytes (hSC-CMs) in advancing the development of more predictive preclinical cardiac safety assessment is now the subject of extended testing in the regulatory communities. This includes proposals such as the Japan iPS Cardiac Safety Assessment (JiCSA) consortium with the objective to validate hiPSC-CM assay for regulatory purposes and HESI's Comprehensive in vitro Proarrhythmia Assay (CiPA) which aims to obviate the need for clinical QT studies. The core components of CiPA will include a mathematical (i.e. in silico) model of cardiac muscle electrical activity based on in vitro ion channel data to predict whether new drugs will cause dangerous changes to heart rhythm (i.e. proarrhythmia). A complementary in vitro hSC-CM assay will be integrated into this process with the aim of confirming or casting doubt on the in silico predictions and to broaden the cardiac safety assessment of the candidate drug to include additional proarrhythmic mechanisms not discoverable by the in silico analysis. This is a very promising development for the acceptance

of hSC-derived models in drug discovery and toxicology, and with increased characterization of these hSC-derived models and validation of their associated assays, they will surely grow in prominence.

The majority of this book is focused on differentiated tissues, but we begin the protocol chapters with a high-throughput screen designed to predict embryonic-foetal developmental toxicity using hESCs (Chap. 2, Kameoka and Chiao). One of the first applications of pluripotent stem cells in toxicology was the mouse embryonic stem cell test (mEST), which has been deemed of sufficient value for consideration in regulatory acceptance and submission documents. Here, Kameoka and Chiao describe an improved human embryonic stem cell test (hEST) thus avoiding the extrapolation of responses from animals to humans.

Over half of the chapters in this volume are focused on cardiotoxicity applications. The underlying reason for this bias towards hSC-CM assays is severalfold. Firstly, the cardiomyocyte differentiation process was one of the first to be characterized (hESC-CMs in 2003 and hiPSC-CMs in 2009) and can now be employed to manufacture these cells on an industrial scale suitable for screening applications. Secondly, a human primary model is not readily available (e.g. unlike for hSC-hepatocytes), so there is a large unmet need from the cardiotoxic field. Thirdly, the hSC-CM model has been demonstrated to add value over and above existing model systems (e.g. the antihistamine, terfenadine, produces the expected prolongation of the cardiac action potential in hSC-CMs at clinical relevant concentrations, unlike the false-negative result observed in both canine and porcine Purkinje fibres even at supra-therapeutic concentrations). Finally, cardiotoxicity is one of the most prevalent forms of drug-induced toxicity. Although the recent regulatory guidelines have been successful in reducing the release of proarrhythmic drugs coming to market, there is a general consensus that the extensive focus on a single ion channel (hERG K^+ channel) has resulted in an overly high attrition rate (false positives) in drug development, prematurely halting the development of otherwise promising candidate drugs. CiPA proposes that safety studies using multiple ion channel effects (MICE) models, such as hSC-CMs, are likely to be more predictive of clinical drug response where compensatory drug actions on one or more other ion channels mitigate the effects due to hERG blockade.

Manual patch-clamp remains the “gold-standard” for probing drug-induced cardiac ion channel effects (see Chap. 3, Renganathan et al.), and an automated method for increasing assay throughput is also described here (Chap. 4, Obergrussberger et al.). However, manual patch-clamp is very labour intensive, and the difficulties translating automated patch-clamp protocols to hSC-derived models have resulted in the CiPA initiative focusing on analogous emerging electrophysiology-based technologies, namely, multi-electrode array (MEA; see Chap. 5, Millard et al., and Chap. 10 Obergrussberger et al.) and voltage-sensitive optical probes (i.e. genetically encoded voltage indicators, GEVIs (see Chap. 6, Dempsey et al.), and voltage-sensitive dyes, VSDs (see Chap. 7, Kettenhofen)). These techniques present their own strengths and limitations as discussed in the relevant chapters.

Not all cardiotoxicity can be observed by changes in hSC-CM excitability with the aforementioned electrophysiology-based assays. Full and efficient assessment of new drug development liabilities must take a holistic account of both the structural and functional aspects of cell biology. The relative complexity of stem cell-derived models makes them applicable to surveying a wide range of mechanisms whereby a new chemical entity may perturb cell function. Accordingly, this has sparked the development of a diverse range of innovative analytical platforms with the potential to probe previously inaccessible features of cell function (Fig. 1).

Cardiomyocyte excitability (i.e. an action potential) initiates calcium release into the cell and is subsequently removed from the cytoplasm prior to the next contraction event.

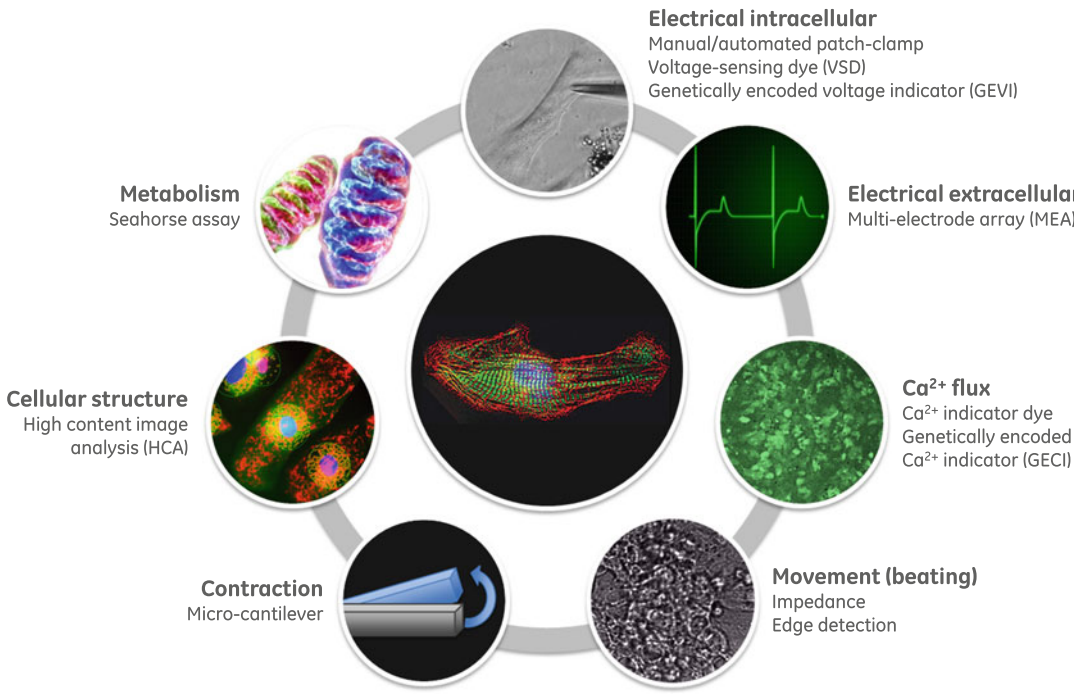

Fig. 1 One cell to bind them all: bridging analytical platforms. As well as providing a more relevant nonclinical model for in vitro toxicity testing, hSC-derived models have the potential to bridge analytical platforms and in doing so provide an integrated model for probing many possible causes and mechanisms of toxicity. The example of hSC-cardiomyocytes (*centre*) and some of the possible associated assays are illustrated here

Compound-induced effects on these calcium transients can be measured with high-throughput calcium imaging platforms as either the mean response of the hSC-CM monolayer (see Chap. 7, Kettenhofen) or with single-cell resolution (see Chap. 8, Pfeiffer et al., and Chap. 9, George et al.). In turn, this calcium signal is converted into the mechanical contraction of the cardiomyocyte. The physical movement of a layer of beating cardiomyocytes can be continuously monitored with the impedance assay (see Chap. 10, Obergrussberger et al.) or image-based edge detection of a beating spheroid (see Chap. 11, Zuppinger et al.). Measuring this downstream hSC-CM movement can be advantageous since compounds can perturb the cell's contractile machinery, without impacting its electrophysiology, e.g. the myosin II inhibitor blebbistatin. These platforms, however, do not measure the direct force of contraction, which would be advantageous when screening for unwanted compound-induced changes to the strength of cardiac contractility (i.e. inotropic effects). This requirement is addressed in Chap. 12 (Oleaga et al.), where a novel microcantilever-based device is employed to detect perturbations in the force of hSC-CM contractions.

In addition to altering the acute mechanical function of the heart (functional toxicity), cardiotoxicity can also occur due to morphological damage to cardiomyocytes, damage to intracellular organelles, or loss of cardiomyocyte viability (structural toxicity), resulting in cardiomyopathy and heart failure. Methods for screening for compound-induced changes to cell morphology are described in Chap. 13 (Roquemore et al., high-content image analysis, HCA) and Chap. 14 (Kriston-Vizi et al., hypertrophy).

Human SC-neurons similarly offer a novel method of screening for neurotoxins. The MEA assay can be employed to predict neurotoxic risk associated with drugs or compounds present in the environment by monitoring subtle perturbations to the spontaneous firing patterns of hSC-neuronal cultures (e.g. insecticides; see Chap. 15, Kraushaar et al.).

Hepatotoxicity is a major cause of drug attrition, and consequently, in vitro liver-based assays are an integral part of preclinical safety assessments. Primary human hepatocytes are currently the model of choice but are limited by donor-to-donor variability and the short period of time they are functional in culture (i.e. they are unsuitable for assessing the effects of prolonged compound exposure). Human SC-hepatocytes could potentially address these concerns, but their implementation in toxicity assays has been hindered largely due to difficulties in obtaining a mature metabolic phenotype. The expression of CYP3A4, the most abundant cytochrome P450 in the liver, is expressed at lower levels in hSC-hepatocytes compared with primary human hepatocytes. This is of concern since CYP450-dependent formation of toxic metabolites is a cause of drug-induced liver injury. That said, promising hSC-hepatocyte data has emerged from methods attempting to replicate the cellular microstructure of the liver (see Chap. 16, Ware and Khetani).

With the first commercially available hSC-derived models, there was an initial drive to produce >95% "pure" cell models. The rationale behind this approach was that it would be preferable if drug-induced effects observed in biochemical assays could be assigned to a single-cell type. However, recent findings suggest that the presence of stromal (e.g. fibroblast) cells in the culture of interest adds functionality and ultimately increases predictivity. The precise mixing of different highly pure cell types with stromal cells now allows the end user to titre the performance of the model to achieve the desired functionality (see Chap. 11, Zuppinger, for hSC-CMs; and Chap. 16, Ware and Khetani, for hSC-hepatocytes). The advent of technologies that facilitate self-assembling 3D spheroids and self-ordering patterns of cells in 2D culture has helped limit the inevitable increase in assay set-up time and cost associated with the added complexity of these co-culture models.

For the first time, this volume brings together a diverse collection of stem cell-derived model-based toxicity assays, from those routinely used to those deemed to have considerable potential. Key opinion leaders from academia and industry have been invited to contribute their preferred assay protocols with accompanying rationale and example output data. Our goal is to enable adoption of these protocols in laboratories that are interested in entering the field as well as to facilitate the transfer of best practices between laboratories that are already actively pursuing these technologies. The use of stem cell-derived models in safety pharmacology and toxicology is in their infancy, but their potential for improving risk assessment will inevitably drive the development of even more innovative methods to probe toxicity.

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Chapter 1

Stem Cell-Derived Models for Safety and Toxicity Assessments: Present and Future Studies in the "Proclinical Space"

Gary Gintant and Stefan Braam

Abstract

The promise of human, stem cell-derived models for safety and toxicity assessments remains great. Using such preparations it should be possible to provide preclinical assessments of drug effects with human-derived cells and engineered tissues, creating a new "proclinical" paradigm to study human responses without administering drugs to human volunteers or patients. Along with this promise come challenges related to more fully characterizing, standardizing, and understanding these novel preparations, developing the experimental platforms necessary for efficient and reproducible studies, and validation studies demonstrating overall utility of various models. This chapter describes some issues encountered with the development of human-induced stem cell-derived cardiomyocytes for safety and toxicity studies with evolving drug candidates, along with a discussion of the role of future proclinical studies as part of an integrated package of more traditional safety and toxicology assessments.

Key words Preclinical studies, Translational, Stem cell-derived cardiomyocytes, Proclinical studies, CiPA, Phenotypic assays, Drug screening

1 The Promise of Human-Induced Pluripotent Stem Cell-derived Cardiomyocytes

Over the past two decades, two seminal scientific breakthroughs, namely, (1) the first report of long-term culture of human *embryonic* stem cells by Jamie Thomson [1] and (2) the discovery of human-*induced* pluripotent stem cell-derived cells (iPSCs) by Shinya Yamanaka [2], led to the birth of a completely new field of translational science focused on the derivation of personalized fully functional human cells (both healthy and diseased) of many different organs. Many major technical advances followed quickly. In particular, major improvements have been made in the process of deriving these cells in culture and the development of reproducible differentiation strategies to produce functional cells like cardiomyocytes, neurons, and hepatocytes in numbers and purities amenable for functional assays. These advances sparked interest in the

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development of novel preclinical in vitro testing strategies (to evaluate drug safety and efficacy) as well as the development of novel cellular therapies for human diseases.

The functional cell types derived from "healthy" (representing non-diseased states) iPSCs in combination with novel assay strategies have now found their way to the safety/ADME/toxicology departments of biopharmaceutical companies and CROs where they are being used to study effects of evolving drugs in human cellular models in the preclinical R&D space. Besides being useful for the generation of healthy cells, this technology is very amendable to the generation of "disease-in-a-dish" models. Congenital diseases are at the forefront of these applications, in part because existing genome-wide association datasets for many diseases enable functional assessment in experimental in vitro human models. Furthermore, rapid advances in genome engineering now allow the construction of isogenic control cell lines which increase confidence that observed cellular phenotypes are directly correlated to the genetic variant of interest. In particular, in the case of cell autonomous diseases like cardiac arrhythmias, this technology is exceptionally well placed to study molecular mechanisms that drive disease progression and putative targets for reversal of disease symptoms. It has been demonstrated in many articles that arrhythmic phenotypes can be observed in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) derived from LQT patients [3], a concept inconceivable a decade ago. In conclusion rapid advances in iPSC biology have resulted in novel in vitro models that are already impacting today's drug discovery process.

Most chapters in this book describe platforms and methodological approaches used or being developed for in vitro stem cell human-derived cellular models. It is important to not only understand the strengths and limitations of these various methodological approaches but also to understand the strengths and limitations of the preparations themselves. Both components (preparations and methodological approaches) will define the utility, best applications, and adoption of the models. This chapter will discuss some general principles and concepts regarding the use and translation of phenotypic models employing stem cell-derived preparations for drug discovery and safety, as well as considerations regarding the influence (and promise) of evolving stem cell-derived therapies. While the focus in this chapter may be on cardiomyocytes, comparable issues are applicable with neuronal and hepatocyte models, two areas of intense interest. Finally, while the perspectives and opinions in this chapter are centered primarily on the use of human-derived models for evaluating drug safety and toxicity, these concepts are also applicable to the use of these preparations as disease models in drug discovery.

2 Understanding The Preparations

For decades, biomedical and pharmaceutical researchers have used model systems to study human biology, development, disease, and drug therapy. In particular, model organisms are widely used to investigate treatments and potential causes of human disease when experiments directly on humans are not feasible or are unsafe and therefore unethical. Examples of commonly used laboratory model systems include the fruit fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, the rat *Rattus norvegicus*, and the mouse *Mus musculus*. Largely because of the ability to perform reverse genetics, the mouse has evolved as the favorite model to study human genes and disease processes [4]. Although all of these model organisms have their own specific advantages and disadvantages, they have one major disadvantage in common, namely, they are not human and thus may not possess systems similar to humans. Care must always be taken when extrapolating from any model system to humans.

3 The iPSC-Derived Cardiomyocyte Model

Ideally, human iPSC model systems overcome this major "translational" issue and fulfill the main criteria of an experimental system: simple, idealized, readily accessible, and easily manipulated [5]. With experimental animal models, it is clearly more challenging to study mechanisms that may involve multiple cellular systems and multiple organs, where multiple interactions modulate and define the cellular phenotype through autocrine, paracrine, and endocrine/exocrine factors, as well as physical/mechanical characteristics of the microenvironment and mechanotransduction (see section on Maturation, below). However, processes "at the cellular level" like transcriptional regulation, proliferation, differentiation, cell migration, and specific cellular functions (e.g., electrophysiology and contractility) are very well suited to being studied in human-derived in vitro cellular models [6–8].

Despite higher-level similarities, it remains crucial to recognize cellular differences between acutely isolated primary cells and stem cell-derived cardiomyocytes used in in vitro models. For studies involving ex vivo-derived cells and tissues, nature provides the necessary quality control standards, as the preparations were properly functioning in (presumably) a healthy integrated animal. Consider adult rat papillary muscles as an example. Given identical experimental protocols and reagents, studies conducted with adult rat papillary muscles harvested from a particular strain of healthy rats today would be expected to provide the same experimental result as those generated some 50 years ago. Assuming standardized (and