

Annex 1. The potential for cardiomyocytes in predictive toxicology

Current issues in drug-induced cardiac toxicity (not including vascular toxicity) which significantly affect drug safety and drug development fall into two broad categories: cardiac pathology and cardiac electrophysiology. Stem cell-derived cardiomyocytes could contribute to early screening for these effects, to defining species-specific responses, investigating new biomarkers and increasing our understanding of cardiomyocyte physiology.

- There is a basic requirement for greater characterisation of physiology, pharmacology and development of stem cell-derived cardiomyocytes and how they compare to adult cardiac cells. There is also a need for standardisation in methodology or establishment of best practices. Safety Pharmacology studies address the cardiovascular safety of drugs with recommended tests in both *in vitro* and *in vivo* models for effects of drugs on cardiac electrophysiology and haemodynamics. Translation of effects to humans remains a challenge. The use of stem cell derived-cardiomyocytes might contribute to understanding of acute electrophysiological aspects, such as drug-induced alterations in action potential characteristics, as well as medium term effects relating to prolonged drug exposure (e.g. ion channel trafficking). An assessment of the added value of these assays to cardiac electrophysiological screening would be needed, using direct comparisons with current models. Scale-up will require the development of high throughput platforms such as 96-well micro-electrode arrays (MEA), optical action potential high-throughput technology, or high throughput patch clamp. This would require large numbers of cells containing a well-defined and consistent population of cardiomyocytes.
- Drug-induced cardiac pathology findings such as myocardial necrosis are typically not detected until compounds are tested in long-term animal toxicology studies. Effects may be seen in only one species and there is little understanding of how this translates to man. Where effects are directly related to functional changes in the heart, it might be possible to screen for some of these toxicities using human stem cell-derived-cardiomyocytes, which can be cultured for prolonged periods. This would require considerable validation work to characterise the cell line/s, define pathways involved, explore specificity and sensitivity, and gain understanding of long term changes in cultured cells. A number of techniques could be used including proteomics, transcriptomics and metabolomics in addition to the more conventional assays for apoptosis and necrosis. Existing clinical (e.g. troponin) and potential new biomarkers could be explored. As part of the validation, pharmaceutical companies will provide compounds with known *in vivo* cardiotoxicity data for comparative testing in cardiomyocytes. Potential benefits of a cardiomyocyte approach implemented at early stages of drug development could include better profiling of clinical candidates, reduced animal use in toxicology studies, reduced attrition, better understanding or development of new biomarkers which translate to the clinic and potentially safer clinical studies/medicines.

Annex 2. Cardiomyocyte call - Deliverables for the pilot phase

Obtaining cardiomyocytes from hESC lines and establishing functional readouts

This second call in the pilot phase of the SC4SM initiative focuses on evaluating differentiation and isolation protocols to produce cardiomyocytes, particularly with ventricular-like characteristics. Since the appearance of the cardiomyocyte phenotype has been regularly reported for human ESC lines, applicants will likely have achieved this before submission, however submissions proposing novel approaches and technologies to generate cardiomyocytes more effectively and efficiently will also be considered. Thus, the aim of this project to characterise human stem cell-derived cardiomyocytes (hESC-CM), increase their proportion and purity and the reproducibility of cardiac cell differentiation. HESC applications incorporating iPS as one of the derivation routes will also be considered, but applicants must demonstrate that they are already achieving consistent production of cardiomyocytes to a similar level as hESC-CM. Finally and as part of cell characterisation, the cardiomyocytes obtained would have to be assessed for *in vitro* cardiotoxicity and cardiac safety pharmacology testing, with a focus on acute effects and ion channel function.

Drug-induced cardiac adverse events can be divided into two groups, functional and structural effects. Functional electrophysiological effects of drugs are typically mediated by their direct interaction with cardiac ion channels in ventricular myocardium and/or heart conduction system, which may lead to cardiac arrhythmia. Drug-induced degenerative structural effects are mediated by direct myocardial injury mainly resulting from impaired cellular homeostasis and oxidative stress. Development of *in vitro* assays based on the human stem cell-derived cardiomyocytes would provide an opportunity to assess the specific cardiotoxicity end-points early in the drug development process.

1. Derivation and physiological characterization of promising human stem cell-derived cardiomyocyte cell lines for indication of utility in toxicology:

- To obtain hESC-CM with a stable homogenous population of physiologically relevant phenotype with standardized protocols that can be applied reproducibly across a number of lines. Ventricular-like cardiomyocytes are the main interest for cardiotoxicity studies, though future phases may include nodal pacemaker-like cell cultures which can be used to predict drug-induced sinus arrhythmias and atrio-ventricular blocks.
- To apply existing protocols to multiple cell lines to measure and determine reproducibility;
- To assess functionality of the cell phenotype, including ion channel gene/protein expression profiling and ion current measurements (e.g. main inward (Na^+ , Ca^{2+}) and outward (K^+) currents, Na/Ca exchanger, Na/K-ATPase etc).

2. Functional read-outs for validation:

- To establish functional readouts from hESC-CM to validate and utilise differentiated cells in screens, and developing new read-outs to support high throughput toxicology screens. Predictivity should be equal or superior to existing assays for clinical effects;

- To validate differentiated cells against key reference compounds with known adverse effects on cardiac function and structure. This should show the capacity of the cells for arrhythmogenesis and susceptibility to cellular injury. For example, appropriate alteration in current activity or action potential configuration in response to Ca²⁺ antagonists (nifedipine, verapamil) or agonists (Bay K 8644), K⁺/ATP openers, K⁺ channel blockers (e.g. 4-AP, E-4031), Na⁺ channel openers or blockers (e.g. aconitine, lidocain, flecainide). Comprehensive validation of promising cells against a reference set may be continued in the next phase of the SC4SM programme. Electrical parameters of the cells can be measured using conventional or automated patch clamp, optical action potential technology or microelectrode array (MEA) technology. Translational safety biomarkers for toxic myocardial injury may include cardiac troponin, CK-MB and LDH-1/2.

For both objectives, proposals should clearly detail the route to validation, criteria for success, and eventual thoughts around an implementation plan. There is the possibility for member pharmaceutical companies to assist with analytical evaluation and the provision of compound reference tools and comparator cells. This Annex serves as an illustration of how a scientific plan may be laid out. Applicants may propose to address some or all aspects, including others they may deem appropriate in achieving the objectives of the SC4SM call.

For example, the work can be divided into subprojects:

- a) Modification and adaptation of existing cardiac differentiation protocols to increase proportion of beating embryoid bodies (EBs)/ cardiomyocytes within EBs/ specific cardiac cell types /produce cardiomyocytes in monolayer culture rather than EBs.
- b) Development of protocols to increase the purity of a specific cardiomyocyte cell type (ventricular) using selection criteria.
- c) Characterisation of cells obtained by a qualified set of acceptance criteria for differentiated cardiomyocytes (surface markers, gene expression, protein and metabolic profiles) and functional assessment of the cell phenotype.

In the longer term, maturation of cardiomyocyte phenotype and maintenance in culture would be of interest, however not necessarily addressed in this phase.

Deliverables - timescale and milestones

These are indicative, and applicants should outline realistic milestones depending on the capability of their groups and the scientific approach adopted. For example

Q1-2:

- Confirm characteristics of differentiated cells, for example by antibody marker and RT-PCR methods, and establish assays for cardiomyocyte functional characteristics.
- Development of useful markers and assays as appropriate, e.g. to distinguish cardiomyocytes from (spontaneously active) smooth or skeletal muscle cells and to differentiate between ventricular-like cardiomyocytes and other cardiac cell types such as nodal pacemaker-like cells.
- Establish improved methods of purifying ventricular cardiomyocytes from other differentiated cell types.

Q3-4:

- Demonstrate reproducibility of effective differentiation/purification protocols and increase in proportion of cardiomyocytes, i.e. cardiac or precardiac mesoderm markers, cardiomyocyte markers.
- Carry out functional assessment of the cardiomyocyte cell phenotype in relation to properties for predictive toxicology.
- Carry out early toxicology evaluations.

Annex 3.**Possible markers for the characterisation of hESC-derived cardiomyocyte-like cells****The characterisation of ventricular cardiomyocytes**

A longer term aim within the full 5-year initiative will be to demonstrate that cardiomyocytes/nodal pacemaker-like cells derived from hESCs have the respective phenotype and functionality that is similar to primary human cardiac cells and superior to current standard cell lines (hERG expressing cell lines) or primary animal cardiomyocytes for the assessment of functional electrophysiological safety evaluation.

The following tabulated list might give some guidance towards the characterisation of existing and novel cardiac-like cells, and comparison with primary human or animal cardiomyocytes. A sequential plan of characterisation (stages: I basic phenotype, II cell function, III response to toxic agents) may be employed. This staged approach aims to eliminate unsuitable cell production methods as early as possible, with more promising strategies going forward for further analysis.

Markers: see Table

Loss of markers of undifferentiated state: Oct3/4, SSEA4, Tra-1-81

Phenotype:

- Beating/contractile (visual), sarcomere development

Functional: see Table

- Ion channels (Na⁺, Ca, K) and ventricular action potential with notch and plateau configuration. Absence of I_{kACh}, I_f for ventricular cells. Loss of T-type channel with maturation.
- catecholamine response to increase rate (distinguish from skeletal, smooth muscle). Lack of direct responses on rate via adenosine A1 or muscarinic agonists, with anti-adrenergic responses retained (ventricular phenotype).

Stage	Feature	Assay technology	Examples of markers
I	Morphology	Light microscopy	Contractile activity (spontaneous or paced), organisation of contractile proteins, sarcomere alignment.
I	Protein expression	ICC/ ELISA	cardiac myosin heavy-chain, myosin light chain-2a and -2v, α-actin, cardiac troponin T and I, SERCA2a, phospholamban, RyR, at appropriate times, ion channels and pumps
I	Transcription factors, gene expression	Taqman, PCR	MEF-2, Nkx2.5, Tbx2 and 5, GATA4, cardiac myosin heavy-chain, myosin light chain-2a and -2v, α-actin, cardiac troponin T and I, SERCA2a, phospholamban, RyR,

			SCN5A (INa), KCND3/KCND2 (Ito), KCNH2 (IKr), HCN1, HCN2, HCN4 (If), KCNJ2 (IK1), CACNA1c (ICa,L), KvLQT1(IKs), SLC8A1 (Na/Ca exchanger)
I	Phenotypic stability	Various	Reproducibility of responses within and between hESC-CM cells/clusters over time under assay conditions.
II	Ion channel and exchanger function	Electro-physiology	hERG, I _{Na} , I _f , T and L-type Ca, I _{Ks} , I _{Kr} , I _{KATP} , I _{KAch} , Na/Ca exchanger, Na ⁺ /K ⁺ -ATPase, Ito, Late INa), pacemaker current.
II	Calcium handling	Ca imaging, IHC	RyR, SERCA, force frequency relationship.
III	Response to test compounds	Beating rate, arrhythmia detection	Isoprenaline, dobutamine, carbachol, adenosine, E4301 (HERG blocker), verapamil, nifedipine, lemakalim, 5HT, histamine Dofetilide, d-sotalol, terfenadine, cisapride, pinacidil, amiodarone, zatebradine (If blocker), HMR 1556 (IKs blocker), SEA0400 (Na/Ca exchanger antagonist), ATX II (Late INa opener). Other drugs can be selected from Redfern et al 2003 Cardiovasc Res.