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REVIEW ARTICLE

Human Pluripotent Stem Cells and Drug Discovery: A New Beginning

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ABSTRACT

Human pluripotent stem cells (hPSCs) offer unique opportunities to discover and develop a new generation of drugs. Their ability to differentiate into virtually any cell type renders them a cost-effective, renewable source of tissue-specific cell types capable of predicting human responses towards novel chemical entities. Using these improved *in vitro* models based on physiologically relevant human cell types could result in identifying highly precise and safe compounds, thereby reducing drug attrition rates. Moreover, ability to develop humanised disease models for patient-stratified drug screening makes hPSCs an impeccable tool in translational medicine. In this mini-review we focus on the positives and negatives of utilising hPSC-derived cell types as drug discovery platforms with special emphasis on cardio-, hepato- and embryotoxicity.

Keywords: Human pluripotent stem cells, cardiomyocytes, hepatocytes, drug discovery, cardiotoxicity, hepatotoxicity, disease modelling, embryotoxicity

1. INTRODUCTION

Drug attrition; the gradual reduction of pharmacological compounds while progressing along their development pipeline is the most pressing issue in the pharmaceutical industry over the last few decades. The main reason for drug attrition is the inappropriateness of a product with regard to either efficacy or safety. However, by the time these concerns are identified; new compounds have already entered clinical trials. In 2001, lack of efficacy and safety resulted in the withdrawal of ~ 60% of compounds which entered clinical trials[1]. In 2004, the developmental cost of a marketable product was estimated at an escalating US$ 900 million[2] and at this time, the US FDA had approved 36 new compounds which were later reduced[3] to 20 by 2005. In order to reduce drug attrition rates as well as incurring developmental costs, evoking an *in vitro* humanised predictive system to evaluate chemical entities along their development pipeline is highly desirable.

Animal *in vitro* and *in vivo* test systems are currently being used to evaluate the efficacy and safety of a potential compound[4,5]. However, due to species variation and pharmaco-toxicological differences, animal toxicological test systems are challenging, as results obtained from such models are not ideal representations of what may incur in humans[6-8]. The large number of animals required for comprehensive toxicological testing also poses a significant issue. The requirement for eliminating species variation and the constant pressure to minimise the use of test animals further emphasises the need to develop novel, robust and economical *in vitro* humanised models. This is not to say that human test systems are non-existent. However, human test systems which include immortalised cell lines and primary cell types, do not represent cell types in their natural state with primary cells losing tissue specific functions over time.

The derivation of human embryonic stem cells (hESCs) in the late nineties[9] and induced pluripotent stem cells (hiPSCs) in 2007-08[10,11] ushered in a new era in basic, translational and clinical research. The ability of human pluripotent stem cells (hPSCs) to self-renew and differentiate into virtually any cell type of the three germ layers provides an unparalleled advantage over somatic cell test systems[12,13]. Their unlimited supply and reproducible differentiation[14-17] into desired cell types allows for unique opportunities in drug development[5,18-21]. In this mini-review, we direct our focus on the advantages and disadvantages of utilising hPSC-derived cell types as drug discovery platforms with special emphasis on cardio- and hepatotoxicity; forerunners for drug attrition as well as embryotoxicity.

2. HUMAN PSC-DERIVED CARDIOMYOCYTES

Cardiomyocytes are one of the most fundamental cell types in cardiac research, however severe limitations in obtaining workable human cardiomyocytes significantly hampers their use in pre-clinical drug discovery[22]. Moreover, due to their limited proliferation capacity,

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mature cardiomyocytes are incapable of undergoing long-term culture, requiring the need for repeated isolation of primary tissue necessary for *in vitro* functional analysis. Currently, canine cardiomyocytes are the most widely used pre-clinical model for assessing cardiotoxicity[23]. Apart from being non-human, ethical concerns surrounding the killing of hundreds of dogs annually together with substantial costs are major limitations associated with this test system. Heterologous over-expression systems which include cell lines ectopically expressing specific cardiac ion channels are also utilised as *in vitro* test systems. However, over-expression of a single ion channel in isolation from a complex ion channel network within cardiomyocytes may prove to be disadvantageous. The market withdrawal of Vioxx further emphasises as to why *in vitro* humanised models which can predict efficacy and safety with high precision are mandatory[5,23].

Blockage of the potassium channel hERG which regulates the I_{Kr} current is a key criterion for drug attrition. Proper regulation of I_{Kr} is vital for normal cardiac repolarisation and prevention of QT prolongation. The QT interval represents the duration of ventricular depolarisation and subsequent repolarisation and prolongation of this interval to more than 450ms could trigger life-threatening arrhythmias[23]. Pharmacological compounds such as thioridazine, nilotinib and arsenic trioxide are reported to cause QT prolongation. The FDA has thus recognised QT prolongation as an important variable which needs to be addressed during drug development while the International Conference of Harmonisation (ICH) Expert Working Group (EWG) for drug development has placed risk assessments for delayed ventricular repolarisation as standard procedure of pre-clinical evaluation of new chemical entities[24].

Cardiomyocytes differentiated from hPSCs open a new paradigm in regenerative medicine[25,26], disease modelling[20,27], drug discovery[18-20,28] and understanding developmental phenomena[17,29]. Similar to their adult counterparts, hPSC-derived cardiomyocytes express cardiac genes, structural proteins and ion channels[17,30] as well as exhibit a functional excitation-contraction coupling system which could be modulated through β1/β2-adrenergic and muscarinic receptor agonists[31]. Evaluation of electrophysiological properties could be performed on single cells (patch clamp) or clusters (multi-electrode analysis; MEA) which allow for studying action potential (AP) and field potential duration (FPD) respectively (apart from other parameters). While the ability to assess the effect of a compound on a single ion channel (K^+, Na^+, Ca^{2+}) or complex channel network (AP, FPD) enhances the prediction value of hPSC-cardiomyocytes, the absence of species variation renders these cells an impeccable humanised *in vitro* test system. A number of studies have demonstrated the potential application of hPSC-cardiomyocytes in evaluating pharmacological safety[23,32-36]. For example, while E-4031 (I_{Kr} blocker) and Satalol (class III anti-arrhythmic agent; I_{Ks} blocker) were reported to induce QT prolongation, administration of quinidine (class I anti-arrhythmic agent; I_{Na} blocker) and TTX (Na^+ channel blocker) resulted in a dose-dependent reduction in conduction velocity[18,32].

With the establishment of hiPSC technology, patient-specific cardiomyocytes could be generated, providing unprecedented insight into the molecular mechanisms surrounding disease pathogenesis[20]. Testing of compounds on patient-specific cardiomyocytes would result in a more accurate prediction with regard to efficacy and safety. Currently hiPSC models bearing various hERG mutations have been generated for studying congenital Long QT syndrome 2; a familial arrhythmogenic syndrome characterised by delayed repolarisation which could result in life threatening polymorphic ventricular tachycardia[20,37,38]. While LQTS2 cardiomyocytes derived from patient-specific iPSCs recapitulate the disease phenotype (prolonged QT interval), treatment with ion channel modulators and β-blockers resulted in QT shortening and reduced the incidence of early after depolarisation (EAD) events. Targeting of the proteasomal complex has also proven to be successful in reversing the Long QT phenotype, showcasing the tremendous potential of hiPSCs in drug discovery[20].

Drawback surrounding hPSC-cardiomyocytes is their failure to completely develop a mature phenotype. Irregular sarcomeric organisation[18,31], multi-angular morphologies (spindle, round, triangular), absence of a T-tubule system[31] and the expression of foetal proteins (e.g. SMA, Cx45)[15,39,40] suggest hPSC-cardiomyocytes are structurally more foetal-like in nature. Spontaneous contractions, auto-arrhythmic behaviour and smaller amplitudes of various currents indicate electrical immaturity as well. Based on these structural and electrophysiological disparities, when compared to adult cardiomyocytes, hPSC-cardiomyocytes seem to resemble a 16-week old foetal heart[23,41] which may be disadvantageous during pharmacological evaluations. For example, dose-response studies have shown that hESC-cardiomyocytes respond to isoprenaline at ~10nM[42], which is similar to a failing heart rather than a health one, which responds to ~1-2 nM depending on the age of the individual[43].

When using hPSC-cardiomyocytes as a test system, evaluation of a compound focusses primarily on electrophysiological alterations (e.g. QT prolongation). It would be of utmost importance if hPSC-cardiomyocytes could also provide insight into compounds capable of inducing indirect cardiotoxicity. Treatment with anti-cancer drug trastuzumab (Herceptin); a monoclonal antibody administered for breast cancer resulted in a 4% increase in heart failure and decreased heart function by 10% especially in patients co-administered with anthracyclines[44]. Since these side effects were unforeseen until phase III studies, adequate end-points should be developed to evaluate all-manner of cardiotoxicity (direct and indirect). To circumvent these issues, apart from electrophysiological alterations, the secretion of cardiac troponins and natriuretic peptides into culture medium have been used as indicators of potential cardiotoxicity[45,46]. Improved understanding of candidate biomarkers would

therefore allow for heightened precision in predicting efficacy and safety of a novel chemical entity.

3. HUMAN PSC-DERIVED HEPATOCYTES

Targeting of liver metabolism, thereby modulating interplay between hepatocytes and other organs is considered a viable solution for metabolic ailments. However, more than 40% of new compounds fail their clinical phase trials due to unforeseen pharmacokinetic complications[47], rendering drug-induced liver toxicity a primary reason for attrition. It is estimated that 298 approved drugs cause hepatotoxicity as a side effect and while 6 compounds have been suspended or withdrawn, more than 250 compounds which are associated with acute liver failure still remain in the market[48]. Hepatic death (apoptosis or necrosis) could be induced by a number of cellular factors (depletion of glutathione, binding to lipids and enzymes, direct targeting of mitochondrial complex and immune reactions)[49] with individual risk factors (genetic predisposition, differences in liver metabolism and environment) accounting for unforeseen complications[50].

Isolated human hepatocytes are considered suitable *in vitro* test systems for evaluating drug metabolism and toxicity, yet they are associated with severe limitations[51]. Apart from reduced proliferative capacity, primary hepatocytes retain physiological characteristics only for limited periods as most functions are lost during culture. Moreover, variation between donors (genetic polymorphisms, epigenetic status, health, diet and medications) makes unbiased evaluation and interpretation challenging. As with cardiomyocytes, species variation strongly hampers the utilisation of animal hepatocytes as test systems and while human hepatocellular carcinoma cell lines may be considered as alternatives to primary hepatocytes, these cell types exhibit partial hepatocyte characteristics[47,52]. Humanised *in vitro* models capable of mirroring the complexity of hepatocytes with accurate prediction of candidate compounds is therefore highly desirable.

Human PSCs are an ideal source for obtaining functional hepatocytes and as reports suggest, more than 20 research groups are actively involved in attempts to optimize differentiation as well as maturation of hepatocytes. Combinations of growth factors and signal activators[53-61], co-culture techniques[62-64] and various matrices[65-67] have been implemented in attempts to induce hepatic differentiation. Once generated, hPSC-hepatocytes are reported to exhibit metabolic functionality such as glycogen storage, albumin secretion, urea synthesis, uptake and release of indocyanine green, AAT secretion and LDL uptake[53,57,67-69]. Regardless of their metabolic functionalities, the most critical aspect for potential application of hPSC-hepatocytes in safety pharmacology and toxicology is their ability to metabolize drugs using phase I and II enzymes which facilitate drug metabolism[5]. Data based on hPSC-hepatocytes demonstrating phase I and II enzyme activity is still rudimentary with various discrepancies having been reported[54,59-61,69-71]. The differentiation protocol implemented, the hPSC line used, the quality of the standardised adult hepatocytes have been postulated as reasons for these reported discrepancies.

Despite many protocols being made available for the generation of hPSC-hepatocytes, down-stream cellular characterisation is based solely on gene and protein expression levels[62,72]. The identity of hPSC-hepatocytes therefore remains an issue as non-hepatic cell types are also derived during the differentiation process. Development of a cost-effective, universally robust differentiation protocol would greatly assist in circumventing such issues. Furthermore, generation of hiPSCs from various ethnic groups and polymorphic variants as well as from patients with specific metabolic diseases would allow for population-based compound evaluation and influence the development of patient-stratified compounds.

4. HUMAN PSCS AND EMBRYOTOXICITY

Adverse effects caused by compounds on the reproductive system or developing embryo (growth retardation, malformations or death) is referred to as reproductive toxicology[73]. While it would be ideal to model the reproductive cycle *in vitro*, its severe complexity renders it virtually impossible to do so. If the cycle were to be broken down into individual segments however, it may be possible to develop a suitable test system. Since this by itself is challenging, evaluation of embryotoxicity is still performed on pregnant animals including rats and rabbits. The large number of animals required for tests, including their offspring makes it a laborious and non-economical test system. As with cardio- and hepatotoxicity animal test systems, species variation would thwart accurate prediction of a given compound. An unfortunate example such as the thalidomide disaster has urged scientific communities to develop alternative methods for testing embryotoxicity, such as cell differentiation assays using either primary cells or immortalised cell lines[73,74].

Initially, mouse embryonic stem cells (mESCs) formed the basis of an innovative *in vitro* test system for screening potential embryotoxic compounds. Mouse ESCs could be differentiated into embryoid bodies (EBs) containing the three germ layers (ectoderm, mesoderm and endoderm), marking the onset of early mammalian embryogenesis, thereby establishing itself as a competent test system[75,76]. When the mESC test (mEST) was compared against two other well-established *in vitro* models; rat whole-embryo culture (WEC) and the micromass (MM) test, in an international validation study funded by ECVAM (European Centre for the Validation of Alternative Methods), a 100% prediction rate in the evaluation of strong embryotoxic chemicals was achieved with all 3 *in vitro* test systems[77]. Acceptance of the mEST along with the WEC and MM test by the regulatory commission was therefore expected[78]; however, the Scientific Advisory Committee (ESAC) of the ECVAM felt that these *in vitro* test systems were not adequate[79]. While several strategies have been implemented in attempts to improve mEST[80-90], a major concern which remains is the species variation as it would be challenging to evaluate or

interpret test results in a human context[91,92].

In an attempt to address species variation, hPSCs have been proposed as suitable models for investigating embryotoxicity[91,93,94]. For instance, it was shown that while retinoic acid (RA) and 13-cis RA (13RA) exerted cytotoxic effects on hESCs, only the former compound induced cytotoxicity in mESCs despite both compounds being well-known agents of embryotoxicity[94]. However, testing for cytotoxicity is not a sufficient end-point to establish reproductive toxicity and hence incorporation of biomarkers to monitor changes in early lineage transcripts and cardiac development has been proposed[91,95]. In comparison to animal models, hPSCs have proven their effectiveness as an embryotoxic test system[96]. For example, reversal of arsenic-induced embryotoxicity by monoisoamyl dimercaptosuccinic acid (MiADMSA) was not only observed in pregnant rats but in hESC-EBs as well. Furthermore, arsenic at non-cytotoxic concentrations of 0.1 ng/ml significantly down-regulated genes representing the germ layers which could be abrogated following MiADMSA treatment. These results suggest that while hPSC-EB test systems may be ideal for assessing embryotoxicity, its high level of sensitivity would assist in development of drugs which could revert or prevent the adverse effects of chemical entities.

While preliminary studies demonstrate an applicability of hPSCs as a viable embryotoxicity test system, certain issues are yet to be addressed. Human PSC lines have been reported to demonstrate lineage bias with propensities towards specific cell types during differentiation[97,98]. Since lineage biases could lead to inaccurate predictions, it would be important to consider such intrinsic properties prior to selecting hPSC lines for compound evaluation studies. Ideally, hESC lines which demonstrate equal bias towards all three germ layers would be the most suitable candidates for such studies. When considering economical costs, commercial availability of high-throughput screening tools like AggreWells™ (StemCell Technologies, CA) capable of generating large numbers of uniformed sised EBs could accentuate the screening process by allowing for evaluation of a large number of chemical entities. Finally, if hESC-based *in vitro* test systems could provide positive/encouraging evaluations on drugs found to be embryotoxic following approval, then perhaps further developments in the field may be stimulated.

5. CONCLUSIONS

While hPSC implementation in therapeutic applications (regenerative medicine and disease modeling) has been extensively discussed over the past decade, only recently their potential in drug discovery is being realistically unravelled. The human origin, unlimited proliferative capacity and differentiation potential of hPSCs offer many advantages in biomedical research. Results obtained from proof-of-concept compound evaluation studies renders hPSCs a valuable tool for predicting efficacy and safety with high precision. For hPSCs to be successfully implemented as a robust drug discovery platform, issues needing to be addressed are (a) cost-effective and reproducible ways to generate purified cell populations for high-throughput screening, (b) development of universal and standardised assays for accurately evaluating toxicity of a chemical entity and (c) forging consensus between the scientific community, industry and regulators in developing guidelines for such evaluations.

Conflict of Interest : None

REFERENCES

1. Laustriat, D.; Gide, J. & Peschanski, M. Human pluripotent stem cells in drug discovery and predictive toxicology. *Biochem Soc Trans.*, 2010, **38**(4), 1051-7. doi: 10.1042/BST0381051
2. Kola, I. & Landis, J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov*, 2004, **3**(8), 711-5. doi: 10.1038/nrd1470
3. Sartipy, P. *et al.* The application of human embryonic stem cell technologies to drug discovery. *Drug Discov Today,* 2007,**12**(17-18), 688-99. doi: 10.1016/j.drudis.2007.07.005
4. Stummann, T.C. *et al.* Report and recommendations of the workshop of the European Centre for the Validation of Alternative Methods for Drug-Induced Cardiotoxicity. *Cardiovasc Toxicol,* 2009, **9**(3), 107-25. doi: 10.1007/s12012-009-9045-3
5. Wobus, A.M. & P. Loser, Present state and future perspectives of using pluripotent stem cells in toxicology research. *Arch Toxic*ol, 2011, **85**(2), 79-117. doi: 10.1007/s00204-010-0641-6
6. Nau, H. Correlation of transplacental and maternal pharmacokinetics of retinoids during organogenesis with teratogenicity. *Methods Enzymol,* 1990, **190**, 437-48. doi: 10.1016/0076-6879(90)90050-B
7. Nau, H. Embryotoxicity and teratogenicity of topical retinoic acid. *Skin Pharmacol,* 1993, **6**(1), 35-44. doi: 10.1159/000211162
8. Tzimas, G. *et al.* Identification of 9,13-dicis-retinoic acid as a major plasma metabolite of 9-cis-retinoic acid and limited transfer of 9-cis-retinoic acid and 9,13-dicis-retinoic acid to the mouse and rat embryos. *Drug Metab Dispos,* 1994, **22**(6), 928-36.
9. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science*, 1998, **282**(5391), 1145-7. doi: 10.1126/science.282.5391.1145
10. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 2007, **131**(5), 861-72. doi: 10.1016/j.cell.2007.11.019
11. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science,* 2007, **318**(5858), 1917-20. doi: 10.1126/science.1151526
12. Ramachandra, C.J. *et al.* Efficient recombinase-mediated cassette exchange at the AAVS1 locus in human embryonic stem cells using baculoviral vectors. *Nucleic Acids Res,* 2011, **39**(16), e107. doi: 10.1093/nar/gkr409

13. Tay, F.C. *et al.* Targeted transgene insertion into the AAVS1 locus driven by baculoviral vector-mediated zinc finger nuclease expression in human-induced pluripotent stem cells. *J Gene Med,* 2013, **15**(10), 384-95. doi: 10.1002/jgm.2745
14. Itskovitz-Eldor, J. *et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med,* 2000, **6**(2), 88-95.
15. Mummery, C. *et al.* Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation,* 2003, **107**(21), 2733-40. doi: 10.1161/01.CIR.0000068356.38592.68
16. Yang, L. *et al.* Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature,* 2008, **453**(7194), 524-8. doi: 10.1038/nature06894
17. Mehta, A. *et al.* Phasic modulation of Wnt signaling enhances cardiac differentiation in human pluripotent stem cells by recapitulating developmental ontogeny. *Biochim Biophys Acta,* 2014, **1843**(11), 2394-402. doi: 10.1016/j.bbamcr.2014.06.011
18. Mehta, A. *et al.* Pharmacological response of human cardiomyocytes derived from viral-free induced pluripotent stem cells. *Cardiovasc Res,* 2011.
19. Mehta, A. *et al.* Pharmacoelectrophysiology of viral-free induced pluripotent stem cell-derived human cardiomyocytes. *Toxicol Sci*, 2013, **131**(2), 458-69. doi: 10.1093/toxsci/kfs309
20. Mehta, A. *et al.* Re-trafficking of hERG reverses long QT syndrome 2 phenotype in human iPS-derived cardiomyocytes. *Cardiovasc Res,* 2014, **102**(3), 497-506. doi: 10.1093/cvr/cvu060
21. Chen, I.Y., E. Matsa, & J.C. Wu, Induced pluripotent stem cells: at the heart of cardiovascular precision medicine. *Nat Rev Cardiol*, 2016. doi: 10.1038/nrcardio.2016.36
22. Ameen, C. *et al.* Human embryonic stem cells: current technologies and emerging industrial applications. *Crit Rev Oncol Hematol,* 2008, **65**(1), 54-80.
23. Braam, S.R., R. Passier, & C.L. Mummery, Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. T*rends Pharmacol Sci*, 2009, **30**(10), 536-45. doi: 10.1016/j.tips.2009.07.001
24. Cavero, I. & W. Crumb, ICH S7B draft guideline on the non-clinical strategy for testing delayed cardiac repolarisation risk of drugs: a critical analysis. *Expert Opin Drug Saf,* 2005, **4**(3), 509-30.
25. Laflamme, M.A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol,* 2007, **25**(9), 1015-24. doi: 10.1038/nbt1327
26. Ja, K.M. *et al.* iPSC-derived human cardiac progenitor cells improve ventricular remodelling via angiogenesis and interstitial networking of infarcted myocardium. *J Cell Mol Med,* 2016, **20**(2), 323-32.
27. Ramachandra, C.J. *et al.* Molecular pathogenesis of Marfan syndrome. *Int J Cardiol,* 2015, **187**, 585-91. doi: 10.1016/j.ijcard.2015.03.423
28. Mehta, A. *et al.* A systemic evaluation of cardiac differentiation from mRNA reprogrammed human induced pluripotent stem cells. *PLoS One*, 2014, **9**(7), e103485. doi: 10.1371/journal.pone.0103485
29. Ramachandra, C.J. *et al.* ErbB4 Activated p38gamma MAPK Isoform Mediates Early Cardiogenesis Through NKx2.5 in Human Pluripotent Stem Cells. *Stem Cells*, 2016, **34**(2), 288-98. doi: 10.1002/stem.2223
30. Ramachandra, C.J. & W. Shim, Evaluation of sarcomeric organisation in human pluripotent stem cell-derived cardiomyocytes. *Methods Mol Biol,* 2015, **1299**: 161-6. doi: 10.1007/978-1-4939-2572-8_13
31. Denning, C. & D. Anderson, Cardiomyocytes from human embryonic stem cells as predictors of cardiotoxicity. *Drug Discovery Today: Therapeutic Strategies,* 2008, **5**(4), 223. doi: 10.1016/j.ddstr.2008.08.002
32. Caspi, O. *et al.* In vitro electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. *Stem Cells Dev,* 2009, **18**(1), 161-72. doi: 10.1089/scd.2007.0280
33. Jonsson, M.K. *et al.* Quantified proarrhythmic potential of selected human embryonic stem cell-derived cardiomyocytes. *Stem Cell Res,* 2010, **4**(3), 189-200. doi: 10.1016/j.scr.2010.02.001
34. Liang, H. *et al.* Human and murine embryonic stem cell-derived cardiomyocytes serve together as a valuable model for drug safety screening. *Cell Physiol Biochem,* 2010, **25**(4-5), 459-66. doi: 10.1159/000303051
35. Otsuji, T.G. *et al.* Progressive maturation in contracting cardiomyocytes derived from human embryonic stem cells: Qualitative effects on electrophysiological responses to drugs. *Stem Cell Res,* 2010, **4**(3), 201-13. doi: 10.1016/j.scr.2010.01.002
36. Pekkanen-Mattila, M. *et al.* Human embryonic stem cell-derived cardiomyocytes: demonstration of a portion of cardiac cells with fairly mature electrical phenotype. *Exp Biol Med (Maywood),* 2010, **235**(4), 522-30. doi: 10.1258/ebm.2010.009345
37. Itzhaki, I. *et al.* Modelling the long QT syndrome with induced pluripotent stem cells. *Nature,* 2011, **471**(7337), 225-9. doi: 10.1038/nature09747
38. Matsa, E. *et al.* Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. *Eur Heart J,* 2011. doi: 10.1093/eurheartj/ehr073
39. Xu, C. *et al.* Characterisation and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res,* 2002, **91**(6), 501-8. doi: 10.1161/01.RES.0000035254.80718.91
40. Kehat, I. *et al.* High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: a novel in vitro model for the study of conduction. *Circ Res,* 2002, **91**(8), 659-61. doi: 10.1161/01.RES.0000039084.30342.9B
41. Satin, J. *et al.* Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes. *J Physiol,* 2004, **559**(Pt 2), 479-96. doi: 10.1113/

jphysiol.2004.068213
42. Brito-Martins, M., S.E. Harding, & N.N. Ali, beta(1)- and beta(2)-adrenoceptor responses in cardiomyocytes derived from human embryonic stem cells: comparison with failing and non-failing adult human heart. *Br J Pharmacol,* 2008, **153**(4), 751-9. doi: 10.1038/sj.bjp.0707619
43. McKinsey, T.A. & E.N. Olson, Toward transcriptional therapies for the failing heart: chemical screens to modulate genes. J Clin Invest, 2005, **115**(3), 538-46. doi: 10.1172/JCI24144
44. Chien, K.R., Herceptin and the heart--a molecular modifier of cardiac failure. *N Engl J Med*, 2006, **354**(8), 789-90. doi: 10.1056/NEJMp058315
45. Dolci, A. *et al.* Biochemical markers for prediction of chemotherapy-induced cardiotoxicity: systematic review of the literature and recommendations for use. *Am J Clin Pathol*, 2008, **130**(5), 688-95. doi: 10.1309/AJCPB66LRIIVMQDR
46. Andersson, H. *et al.* Assaying cardiac biomarkers for toxicity testing using biosensing and cardiomyocytes derived from human embryonic stem cells. *J Biotechnol,* 2010, **150**(1), 175-81. doi: 10.1016/j.jbiotec.2010.06.023
47. Sartipy, P. *et al.* Pluripotent human stem cells as novel tools in drug discovery and toxicity testing. *Drugs,* 2006, **9**(10), 702-5.
48. Suzuki, A. *et al.* Drugs associated with hepatotoxicity and their reporting frequency of liver adverse events in VigiBase: unified list based on international collaborative work. *Drug Saf,* 2010, **33**(6), 503-22. doi: 10.2165/11535340-000000000-00000
49. Russmann, S., G.A. Kullak-Ublick, and I. Grattagliano, Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr Med Chem,* 2009, **16**(23), 3041-53. doi: 10.2174/092986709788803097
50. Chalasani, N. & E. Bjornsson, Risk factors for idiosyncratic drug-induced liver injury. *Gastroenterology*, 2010, **138**(7), 2246-59. doi: 10.1053/j.gastro.2010.04.001
51. Hewitt, N.J. *et al.* Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev,* 2007, **39**(1), 159-234. doi: 10.1080/03602530601093489
52. Donato, M.T. *et al.* Cell lines: a tool for in vitro drug metabolism studies. C*urr Drug Metab,* 2008, **9**(1), 1-11. doi: 10.2174/138920008783331086
53. Baxter, M.A. *et al.* Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening. *Stem Cell Res,* 2010, **5**(1), 4-22. doi: 10.1016/j.scr.2010.02.002
54. Rambhatla, L. *et al.* Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant*, 2003, **12**(1), 1-11. doi: 10.3727/000000003783985179
55. D'Amour, K.A. *et al.* Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol,* 2005, **23**(12), 1534-41. doi: 10.1038/nbt1163
56. Agarwal, S., K.L. Holton, & R. Lanza, Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells,* 2008, **26**(5), 1117-27. doi: 10.1634/stemcells.2007-1102
57. Cai, J. *et al.* Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology, 2007, **45**(5), 1229-39.
58. Duan, Y. *et al.* Differentiation and characterisation of metabolically functioning hepatocytes from human embryonic stem cells. *Stem Cells,* 2010, **28**(4), 674-86. doi: 10.1002/stem.315
59. Hay, D.C. *et al.* Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. *Proc Natl Acad Sci USA,* 2008, **105**(34), 12301-6. doi: 10.1073/pnas.0806522105
60. Brolen, G. *et al.* Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage. *J Biotechnol,* 2010, **145**(3), 284-94. doi: 10.1016/j.jbiotec.2009.11.007
61. Schwartz, R.E. *et al.* Defined conditions for development of functional hepatic cells from human embryonic stem cells. *Stem Cells Dev,* 2005, **14**(6), 643-55. doi: 10.1089/scd.2005.14.643
62. Zhao, D. *et al.* Derivation and characterisation of hepatic progenitor cells from human embryonic stem cells. *PLoS One,* 2009, **4**(7), e6468.
63. Ishii, T. *et al.* In vitro hepatic maturation of human embryonic stem cells by using a mesenchymal cell line derived from murine fetal livers. *Cell Tissue Res,* 2010, **339**(3), 505-12. doi: 10.1007/s00441-009-0906-7
64. Pei, H. *et al.* Lineage restriction and differentiation of human embryonic stem cells into hepatic progenitors and zone 1 hepatocytes. *Tissue Eng Part C Methods*, 2009, **15**(1), 95-104. doi: 10.1089/ten.tec.2008.0234
65. Ishii, T. *et al.* Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells. *Am J Physiol Gastrointest Liver Physiol,* 2008, **295**(2), G313-21. doi: 10.1152/ajpgi.00072.2008
66. Baharvand, H. *et al.* Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. *Int J Dev Biol,* 2006, **50**(7), 645-52. doi: 10.1387/ijdb.052072hb
67. Soto-Gutierrez, A. *et al.* Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric. *Cell Transplant*, 2006, **15**(4), 335-41. doi: 10.3727/000000006783981945
68. Baharvand, H., S.M. Hashemi, & M. Shahsavani, Differentiation of human embryonic stem cells into functional hepatocyte-like cells in a serum-free adherent culture condition. *Differentiation,* 2008, **76**(5), 465-77. doi: 10.1111/j.1432-0436.2007.00252.x
69. Basma, H. *et al.* Differentiation and transplantation of human embryonic stem cell-derived hepatocytes.

Gastroenterology, 2009, **136**(3), 990-9. doi: 10.1053/j.gastro.2008.10.047

70. Moore, R.N. & P.V. Moghe, Expedited growth factor-mediated specification of human embryonic stem cells toward the hepatic lineage. *Stem Cell Res,* 2009. doi: 10.1016/j.scr.2009.04.004
71. Söderdahl, T. *et al.* Glutathione transferases in hepatocyte-like cells derived from human embryonic stem cells. *Toxicology in Vitro,* 2007, **21**(5), 929. doi: 10.1016/j.tiv.2007.01.021
72. Snykers, S. *et al.* In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells,* 2009, **27**(3), 577-605. doi: 10.1634/stemcells.2008-0963
73. Spielmann, H. Predicting the risk of developmental toxicity from in vitro assays. *Toxicol Appl Pharmacol,* 2005, **207**(2 Suppl), 375-80. doi: 10.1016/j.taap.2005.01.049
74. Huuskonen, H. New models and molecular markers in evaluation of developmental toxicity. *Toxicol Appl Pharmacol,* 2005, **207**(2 Suppl), 495-500.
75. Laschinski, G.; Vogel, R. & Spielmann, H. Cytotoxicity test using blastocyst-derived euploid embryonal stem cells: a new approach to in vitro teratogenesis screening. *Reprod Toxicol,* 1991, **5**(1), 57-64.
76. Genschow, E. *et al.* Development of prediction models for three in vitro embryotoxicity tests which are evaluated in an ECVAM validation study. *ALTEX,* 1999, **16**(2), 73-83.
77. Genschow, E. *et al.* The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods. *Altern Lab Anim,* 2002, **30**(2), 151-76.
78. Balls, M. & Hellsten, E. Statement of the scientific validity of the embryonic stem cell test (EST) -- an in Vitro test for embryotoxicity. *Altern Lab Anim*, 2002, **30**(3), 265-8.
79. Spielmann, H. The way forward in reproductive/developmental toxicity testing. *Altern Lab Anim*, 2009, **37**(6), 641-56.
80. Seiler, A.E. *et al.* Use of murine embryonic stem cells in embryotoxicity assays: the embryonic stem cell test. *Methods Mol. Biol.,* 2006, **329**, 371-95.
81. Bremer, S. *et al.* Establishment of an in vitro reporter gene assay for developmental cardiac toxicity. *Toxicol In Vitro.*, 2001, **15**(3), 215-23.
82. Seiler, A. *et al.* Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. *Reprod. Toxicol*, 2004, **18**(2), 231-40.
83. Schmidt, M.M.; Guan, K. & Wobus, A.M. Lithium influences differentiation and tissue-specific gene expression of mouse embryonic stem (ES) cells in vitro. *Int. J. Dev. Biol.,* 2001, **45**(2), 421-9.
84. Spielmann, H. *et al.* The practical application of three validated in vitro embryotoxicity tests. The report and recommendations of an ECVAM/ZEBET workshop (ECVAM workshop 57). *Altern. Lab Anim.*, 2006, **34**(5), 527-38.
85. Noaksson, K. *et al.* Monitoring differentiation of human embryonic stem cells using real-time PCR. Stem Cells, 2005, **23**(10), 1460-7.
86. zur Nieden, N.I.; Kempka, G. & Ahr, H.J. Molecular multiple endpoint embryonic stem cell test--a possible approach to test for the teratogenic potential of compounds. *Toxicol Appl Pharmaco*l, 2004, **194**(3), 257-69. doi: 10.1016/j.taap.2003.09.019
87. Chapin, R. *et al.* Struggles for equivalence: in vitro developmental toxicity model evolution in pharmaceuticals in 2006. T*oxicol In Vitro*, 2007, **21**(8), 1545-51. doi: 10.1016/j.tiv.2006.10.006
88. Murabe, M. *et al.* A novel embryotoxic estimation method of VPA using ES cells differentiation system. *Biochem Biophys Res Commun,* 2007, **352**(1), 164-9.
89. Marx-Stoelting, P. *et al.* A review of the implementation of the embryonic stem cell test (EST). The report and recommendations of an ECVAM/ReProTect Workshop. *Altern Lab Anim,* 2009, **37**(3), 313-28.
90. Peters, A.K. *et al.* Automated analysis of contractility in the embryonic stem cell test, a novel approach to assess embryotoxicity. *Toxicol In Vitro*, 2008, **22**(8), 1948-56. doi: 10.1016/j.tiv.2008.09.008
91. Mehta, A. *et al.* Assessment of drug induced developmental toxicity using human embryonic stem cells. *Cell Biol Int*, 2008, **32**(11), 1412-24.
92. Krtolica, A. *et al.* Human embryonic stem cells as a model for embryotoxicity screening. *Regen Med,* 2009, **4**(3), 449-59. doi: 10.2217/rme.09.13
93. Stummann, T.C.; Hareng, L. & Bremer, S. Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. *Toxicology,* 2009, **257**(3), 117-26.
94. Adler, S. *et al.* Testing potential developmental toxicants with a cytotoxicity assay based on human embryonic stem cells. *Altern Lab Anim,* 2008, **36**(2), 129-40.
95. Adler, S. *et al.* First steps in establishing a developmental toxicity test method based on human embryonic stem cells. *Toxicol In Vitro,* 2008, **22**(1), 200-11. doi: 10.1016/j.tiv.2007.07.013
96. Flora, S.J. & Mehta, A. Monoisoamyl dimercaptosuccinic acid abrogates arsenic-induced developmental toxicity in human embryonic stem cell-derived embryoid bodies: comparison with in vivo studies. *Biochem Pharmacol,* 2009, **78**(10), 1340-9. doi: 10.1016/j.bcp.2009.07.003
97. Osafune, K. *et al.* Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotechnol, 2008, **26**(3), 313-5.
98. Mehta, A. *et al.* Intrinsic properties and external factors determine the differentiation bias of human embryonic stem cell lines. *Cell Biol Int,* 2010, **34**(10), 1021-31. doi: 10.1042/CBI20100283