Stem cell-derived models to improve mechanistic understanding and prediction of human drug induced liver injury

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List of Abbreviations :

DILI, drug-induced liver injury; ADR, adverse drug reaction; SC-HLC, stem-cell-derived hepatocyte-like cell; EPAA, European Partnership for Alternative Approaches to Animal Testing; NC3Rs, National Centre for the Replacement, Refinement and Reduction of Animals in Research; MRM, multiple-reaction-monitoring; CRISPR, clustered regularly-interspaced short palindromic repeats; iPSC, induced pluripotent stem cell

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Abstract

Current preclinical drug testing does not predict some forms of adverse drug reactions in humans. Efforts at improving predictability of drug-induced tissue injury in humans include using stem cell technology to generate human cells for screening for adverse effects of drugs in humans. The advent of induced pluripotent stem cells means that it may ultimately be possible to develop personalised toxicology to determine inter-individual susceptibility to adverse drug reactions. However, the complexity of idiosyncratic drug-induced liver injury (DILI) means that no current single cell model, whether of primary liver tissue origin, from liver cell lines, or derived from stem cells, adequately emulates what is believed to occur during human DILI. Nevertheless, a single cell model of a human hepatocyte which emulates key features of a hepatocyte is likely to be valuable in assessing potential chemical risk; furthermore understanding how to generate a relevant hepatocyte will also be critical to efforts to build complex multicellular models of the liver. Currently, hepatocyte-like cells differentiated from stem cells still fall short of recapitulating the full mature hepatocellular phenotype. Therefore, we convened a number of experts from the areas of preclinical and clinical hepatotoxicity and safety assessment, from industry, academia and regulatory bodies, to specifically explore the application of stem cells in hepatotoxicity safety assessment, and to make recommendations for the way forward. In this short review, we particularly discuss the importance of benchmarking stem cell-derived hepatocyte-like cells to their terminally-differentiated human counterparts using defined phenotyping, to make sure the cells are relevant and comparable between labs, and outline why this process is essential before the cells are introduced into chemical safety assessment.

Prediction of adverse drug reactions in the liver: why it is important, limitations of current in vitro models and how stem cells may prove useful in drug screening

Adverse drug reactions (ADRs) are a significant clinical problem, resulting in considerable patient morbidity and mortality(1) and thus represent a major financial burden on healthcare systems. ADRs also represent a major challenge for the pharmaceutical industry leading to attrition of drugs in development and the withdrawal of drugs post-licensing(2). Amongst different forms of ADRs, the liver is particularly susceptible to drug toxicity; drug-induced liver injury (DILI) is the second highest cause of attrition and accounts for more than 50% of cases of acute liver failure(3).

The principal cause of these high attrition rates is the failure of current preclinical drug testing procedures to effectively predict idiosyncratic DILI in patients(2). This is true for *in vitro* models and even for *in vivo* models - a recent study that related the preclinical assessment of drugs with the occurrence of DILI in the clinic showed that between 38% (Medline database: 269 out of 710 compounds) and 51% (EMEA database: 70 out of 137 compounds) of drugs that subsequently caused liver injury in patients were not predicted from animal studies(4). Concerted worldwide efforts are therefore required to improve the assessment of hepatotoxic risk for new compounds. In Europe, the SEURAT (http://www.seurat-1.eu/pages/cluster-projects/scrtox.php) and MIP-DILI (http://www.mip-dili.eu/) consortia, and in the US, DILIN (http://www.dilin.org/) and iSAEC (http://www.saeconsortium.org/) are attempting to address this issue. The clinical manifestation of DILI indicates that it is a multi-dimensional and multi-faceted disease(5). Indeed, the diagnosis of DILI is largely based upon exclusion criteria(5). Although the use of currently available cell lines and primary human hepatocyte models has been able to correctly classify a number of DILI compounds as hepatoxins(6-9), idiosyncratic DILI is inherently difficult to model in the laboratory, and therefore highly unlikely to be predicted by simplistic screening strategies, often based on single-cell models involving cell lines. Many approaches use liver-derived cancer cell lines, e.g. HepG2 and HepaRG, which may have value for identifying drugs lacking a propensity to cause idiosyncratic DILI (90-95% predictability), but perform less well for positive predictions (50-89%)(9-11). Metabolically-competent freshly-isolated, or cryopreserved human primary adult hepatocytes are still considered to be the gold-standard single cell model of DILI. Nevertheless, human hepatocytes are difficult to source, they are also costly and functionally variable (reflecting variation in the human population), they undergo severe stress during the isolation process and, critically, they rapidly lose key functions when cultured *in vitro*. Moreover, it is important to note that hepatocyte toxicity *per se* is not the sole cause of hepatotoxicity which, in the intact liver, may involve multiple different cell types including lymphocytes and macrophages. Yet it is reasonable to assume from the work of several groups, over many years, that a metabolically-competent hepatocyte will be an essential component of any model of hepatotoxicity *in vitro*. Thus, a robust and reproducible metabolically-competent hepatocyte-like cell derived from directly reprogrammed cells, or from pluripotent stem cells, would represent a major step forward for the development of a new generation of *in vitro* models.

The imperatives of industry and academia are driven by different model requirements. The priority for industry is a cost-effective and scalable high-throughput screening model that has direct input into ‘go/no go’ decision making during drug development, whilst academic scientists are driven by the need to understand hepatic physiology and the mechanistic basis of DILI. Hepatocytes derived from stem cells can, however, be central to both of these objectives. Whilst significant progress towards a functional hepatic phenotype has been made, it is clear that stem-cell-derived hepatocyte-like cells (SC-HLCs) still fall well short of recapitulating the full mature hepatocellular phenotype(12-15).

Because of the importance and likely impact of developments in this field, scientists with expertise in preclinical and clinical hepatotoxicityand complex and novel forms of *in vitro* cell culture, representing industry, academia and regulatory bodies, assembled at a workshop at the University of Liverpool, under the auspices of the European Partnership for Alternative Approaches to Animal Testing (EPAA) (http://ec.europa.eu/growth/sectors/chemicals/epaa/index_en.htm) and the MRC Centre for Drug Safety Science (https://www.liverpool.ac.uk/drug-safety/). The purpose of the workshop was to specifically explore the application of stem cells in hepatotoxicity safety assessment, and to make recommendations for the way forward. This workshop follows the

EPAA/NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) (https://www.nc3rs.org.uk/) “Stem Cells in Safety Testing Forum” workshop that took place in 2013, with a mandate to provide a platform for permanent dialogue between research groups, to share experiences, problems, successes and opportunities.

Current challenges in the use of stem cell-derived hepatocytes in the safety assessment of new chemical entities

It is clear from a large number of studies(13, 14, 16-47) (see Table 1) that hepatocytes generated from stem cells are not currently sufficiently mature to emulate an adult primary human hepatocyte, and that these cells are probably closer in phenotype to a fetal hepatocyte(12). Many studies using SC-HLCs purport to demonstrate a hepatocyte-like phenotype but do not actually incorporate a physiologically-relevant benchmark (e.g. freshly-isolated human hepatocytes) and a non-physiologically-relevant benchmark (e.g. HepG2 cells); in addition, often very few markers of the hepatic phenotype are used and studies do not always employ quantitatively-relevant assays (e.g mass spectrometry). Thus, inadequate benchmarking has hampered the field and there is likely significant value in identifying a common framework that might allow end users to readily interpret cell phenotype.

Despite the challenges in generating mature hepatocytes, SC-HLCs have recently been shown to retain the cytochrome P450 (CYP) expression profile (specifically CYP2C9 and CYP2D6) of the donor hepatocyte(48, 49), yielding metabolism-specific toxicity for CYP2C9 (benzbromarone) and CYP2D6 (tamoxifen). This is highly relevant as the CYPs are key enzymes of Phase I drug metabolism, that play a key role in the chemical functionalization and eventual elimination of drugs from the body, but which also can yield significant intracellular concentrations of chemically reactive metabolites, leading to cellular and tissue damage of the liver, and therefore DILI (for a review of this area, see Park et al, 2011(50)).

The recent studies outlined above (48, 49) are particularly important as they suggest that modelling some forms of DILI (such as that elicited by benzbromarone or tamoxifen) using stem cell-derived hepatocytes may be possible, and that ultimately the challenges to generating a fully mature HLC will not always be insurmountable.

We consider that there are at least three major challenges to producing mature, physiologically- and pharmacologically-relevant hepatocytes from stem cells:

- Stem cell-derived hepatocytes must mimic several years of development *in vivo*.
- Like primary hepatocytes, the stem cell-derived hepatocyte phenotype is unstable currently in culture(51).
- At the moment, it is difficult to emulate the complexity of the liver, with its unique blood supply and exposure to relevant concentrations of intestinal products and nutrients *in vitro.* Development of three-dimensional culture systems that employ co-cultivation of all cell types found in the liver acinus is likely to be required if we are to recapitulate the liver *in vitro*(51, 52). Following on from this, it is important to remember that a hepatocyte is not a single entity but varies functionally according to the hepatic zone in which it is located. The consequence of this is that some hepatotoxins induce hepatocellular damage in a zone-specific manner and this has not yet begun to be addressed meaningfully in the stem cell field, as we focus our attempts on improving basic functional maturity of the SC-derived cells, but it will need to be considered.

Despite these challenges, there are many promising leads in development, e.g. the discovery of several small molecule inducers of the hepatic phenotype(53), and the finding that microbial-derived secondary metabolites to which immature hepatocytes are likely to be exposed to post-partum may induce a significant increase in maturity. A further paradigm comes from the exploitation of SC-HLCs for demonstration of efficacy; specifically, for the reversal of the hepatic alpha1-antitrypsin-deficient phenotype, shown by Yusa et al(54). This study demonstrated restoration of alpha1-antitrypsin activity was possible on a "sufficiently" mature background, rather than one that was necessarily fully mature and identical to a freshly-isolated adult hepatocyte. Furthermore, a recent study by Ware et al(55) suggests that DILI detection is possible using SC-HLCs in micopatterned co-cultures, in which cells mature to significant levels. It is worth remembering that the hepatocyte exhibits more individual functions (>500) than any of the other ~200 terminally differentiated cell types in the human body. Therefore it is perhaps not surprising that this cell is amongst the most challenging to mature, and we should still continue to explore the utility of hepatocyte-like cells as prototypes rather than await the final "product".

Lessons learned from the use of stem cell-derived cardiomyocytes in detecting cardiotoxicity

A parallel example, from which lessons can be learned, comes from the use of stem cells in the assessment of drug-induced cardiotoxicity – a primary cause of drug attrition. Cardiotoxicity, specifically QT prolongation, has already been successfully modelled using such cells(56-58). In comparison, there is only very recent evidence that SC-HLCs are able to recapitulate hepatotoxic events(49, 55). The difference between successful application of cardiac models compared with hepatic models may reflect the relative specificity of some forms of drug-induced cardiotoxicity, in contrast with the rather pleiotropic and diverse manifestations of hepatotoxicity, at the molecular, cellular, and tissular level(59). Cardiotoxicity often arises due to drug-induced electrical perturbation of the cell interfering with its contractile function(60). Here, the stem cell-cardiomyocyte model provides advantages over recombinant tumour models. Thus, the impact of drugs that cause simple single ion channel or complex multi-channel perturbation can be related to cardiomyocyte arrhythmias and abnormalities in contractility(61). In hepatotoxicity, however, there are myriad factors required to recapitulate toxicity, especially idiosyncratic toxicity where the immune system is also implicated. This is compounded by inter-individual variation in expression of xenobiotic metabolism and transporter proteins in addition to the chemistry of each drug.

Whilst protocols to differentiate stem cells towards cardiomyocytes generate cells that are not fully mature(61), these cells can recapitulate some facets of the cell phenotype required to produce specific forms of cardiotoxicity. This has prompted major international efforts to search for methods to further mature stem cell cardiomyocytes. Each incremental improvement made towards progressing the compliment of ion channels, regulatory pathways and structural proteins to the complete sets found in adult cells will dramatically increase the utility of stem cell cardiomyocytes. The demonstration that specific toxicological phenotypes can be mimicked by stem cell-derived cardiomyocytes allows the cell model to be considered “fit-for-purpose”. This raises the notion of using stem cell-derived hepatocytes that may be sufficiently mature for a specific toxicological assessment even though the cells may lack the full hepatic functionality with respect to drug metabolism, transporter expression etc. For example, where one or two cytochrome P450s (P450s), some relevant phase II enzymes, such as the glutathione transferases and UDP-glucuronyl transferases, and some Phase III proteins (influx and efflux transporters) are expressed at a set and reproducible % of a “typical” human hepatocyte, this cell may in some cases represent a significant and useful model in understanding specifically drug metabolism and possible metabolism-dependent toxicity.

The Importance of Phenotypic Characterisation

For the field to continue to move forward and develop liver cell models that are useful in prediction and mechanistic understanding of DILI, it is essential that the SC-HLCs are properly benchmarked against currently used and relevant human cells, especially fresh primary human hepatocytes and HepG2 cells (see Table 1 and Figure 1). Moreover, the phenotype of the HLCs must be as reproducible as possible, and they should be fully characterised, particularly with reference to the pharmacological phenotype (using a defined panel of training compounds). It is also important that the cell model can provide a static point of reference that can be used to ascertain if real progress is being made. When assessing novel models of hepatotoxicity it is important to use functional assays employing quantitative mass spectrometry whenever possible, as this is now being routinely employed (48, 62, 63)in order to determine the true phenotype of the model. A global proteomic analysis however may be the most appropriate way to characterise the cells, as this would represent a broad visualisation of the physiological phenotype of the cells. Similarity to freshly-isolated hepatocytes/tissue can be established through proteomics and targeted multiple- reaction-monitoring (MRM)-based mass spectrometric analysis of key proteins, such as CYP450s, transporters and intracellular signalling molecules and metabolic and cellular uptake profiles determined. Developments in mass spectrometric technologies mean that it is now possible to analyse small panels of proteins (for example 10-20 transporters or P450s) using MRM, in order to quantify proteins per cell at an *absolute* level*(64)*. This would ensure valid comparisons between currently used models and cells, as well as cells that are developed in the future. Given the inherent deficiencies in a transcriptomic-only approach, which are well-illustrated in a recent landmark paper reporting only a 39% correlation between mRNA and protein at a global level(65), measuring mRNA levels is not recommended for cell characterisation purposes.

As part of a comprehensive assessment of HLC phenotype, recent developments in the field of hepatocyte-selective translatable biomarkers (e.g. miR122(66)) might allow us to translate the response to chemicals between humans, model organisms and cells including SC-HLCs and it is likely that additional novel and selective biomarkers will be identified in the future using models such as SC-HLCs. This is an important area for industry which requires selective and translatable biomarkers of liver injury to monitor potentially hepatotoxic compounds in the clinic.

The recently developed concepts of adverse outcome pathways and points of departure(67) in the field of systems toxicology should also be considered in the context of phenotyping the response to chemical exposure of hepatocyte-like cells that express relevant proteins and pathways. To this end, cells expressing genetic reporters for key adaptive pathways such as Nrf2, PXR andNF-κB will be useful as a means for understanding the earliest events in the biological response to a drug(68-70). However, it is imperative that we develop ways to bridge our findings from these molecular investigations to what actually occurs in DILI in humans –the development of novel bridging biomarkers that allow extrapolation from *in vitro* test system to man will be invaluable in this endeavour. Another important development in relation to hepatocyte genotype and phenotype in DILI is the derivation of SC-HLCs with specific polymorphisms relevant to drug toxicology. Of particular interest in this regard is the developing use of CRISPR technology in SC-HLCs to edit, for example, genes relevant to drug metabolism and toxicity thereby providing a wild type cell and an almost identical cell with an alteration in drug metabolism and toxicological responses, respectively.

Finally, phenotypic characterisation may be assisted by a better understanding of the mechanisms contributing to de-differentiation or loss of phenotype. Consideration of the cellular complexity of the liver and the functional sophistication of a hepatocyte makes it unsurprising that the maintenance of a fully functional hepatocyte in culture is difficult to achieve(71). The cells have been removed from their neighbouring hepatocytes, disrupting their gap junctions and tight junctions which are important for their phenotype, as well as their juxtaposed non-parenchymal cells, which may also be responsible for the differentiated hepatocyte phenotype(72, 73). Dedifferentiation is not a unique process to the liver; when cardiomyocytes are cultured, they also lose some of their *in vivo* phenotype, e.g. the t-tubules are lost, glycogen is accumulated and chromatin becomes dispersed *in vitro*(74). However, the key difference between hepatocytes and myocytes is the importance of the metabolic phenotype with respect to drug toxicity, and it is this function – particularly the phase I CYP450 capacity – that is most rapidly and profoundly depleted(71, 75) - and it is also this function, at a defined proportion of the activity present in human liver, that is essential in any *in vitro* model of a hepatocyte

One area of research that could have a significant impact on attempts to re-establish a functional hepatocyte from stem cells, is the investigation of the precise cellular mechanisms underlying the de-differentiation process that occurs in hepatocytes once they have been removed from the liver. Whilst the factors driving de-differentiation may not be identical to those that drive differentiation, it is likely that one or more pathways and processes uncovered through research into de-differentiation will be amenable for testing in differentiation experiments. If it is not understood how to maintain the dynamic and sophisticated machinery of a fully mature hepatocyte in vitro, it is likely to be difficult to capture the same phenotype in a stem cell-derived cell grown under similar conditions.

Summary and recommendations

- DILI is a complex, multi-dimensional disease, with variable phenotype between individuals, even for a single drug. There is essentially no ideal *in vitro* or *in vivo* model that recapitulates all of the potential features of this injury.
- The aspiration of the field is a “perfect” mature hepatocyte as it exists in a liver - this has not yet been achieved. Until it is, hepatocyte-like cells with known, quantifiable and reproducible proportions of the function of two widely-used standards, i.e. primary fresh human hepatocytes, and HepG2, will be valuable biological models to explore the physiological, pharmacological and toxicological response of hepatocytes to drug exposure.
- These “immature” cells should be explored as models of chemical perturbation using genetic reporters and biomarkers, with continual effort to relate findings to human DILI.
- Global proteomic analysis aligned with biological pathway analysis may be the most appropriate way to characterise HLCs – a small targeted panel of proteins will also help to compare cells for key proteins and functions using absolute quantitation by mass spectrometry. Crucially, this will advance the field by avoiding over-reliance on a small panel

of liver proteins, such as albumin, that may not be representative of a fully mature and functioning liver cell.

- It is likely that niche creation *in vitro*, deploying enhanced matrices(13) and even 3D bioprinting(76) , and incorporating other cell types such as endothelial cells(76, 77) and Kupffer cells(78) *inter alia*, will mature and support hepatocyte function.
- A small panel of chemical benchmarks will be needed to probe the physiological, pharmacological and toxicological function of the cells, only once they have been properly phenotyped. There is little point in exposing HLCs to chemicals chosen as hepatotoxins in man unless we fully characterise the cells.

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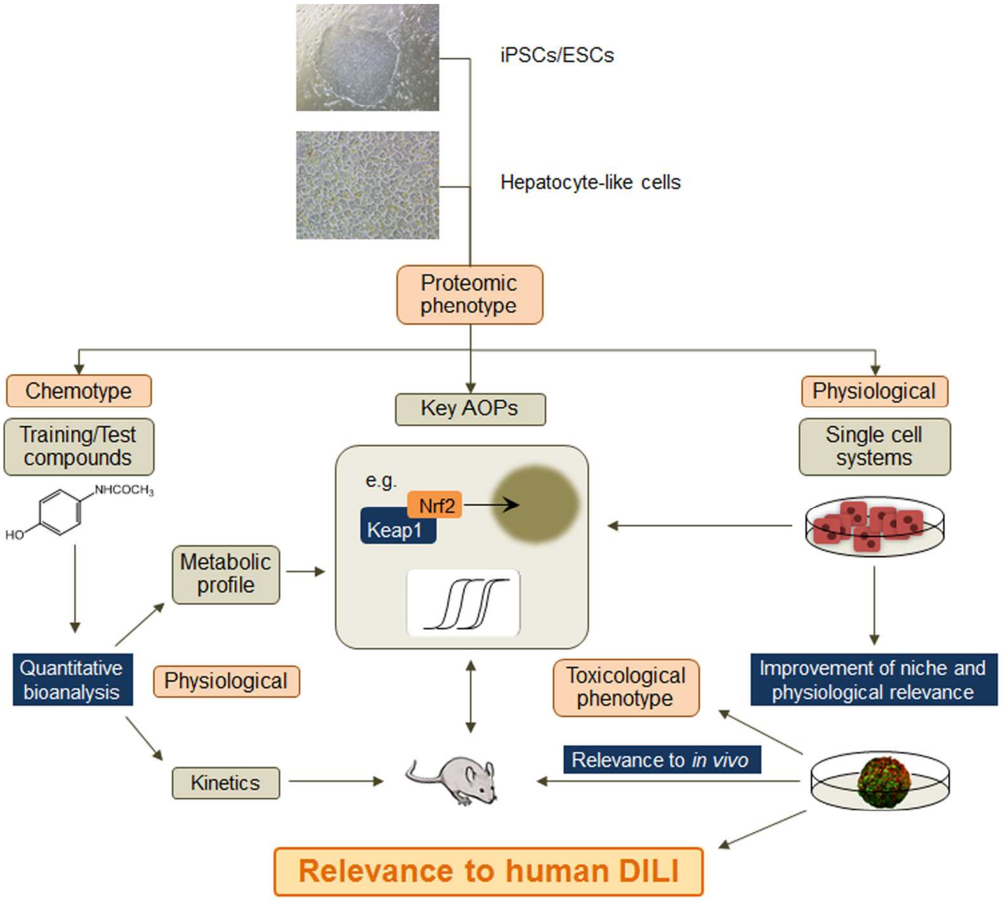
Table 1. Summary of studies post-2007 of HLC-derivation from human pluripotent stem cells (adapted from Table 1 and Table 2, Kia *et al*(13) with modification). Note the limited number of Phase 1 and 2 phenotyping markers generally employed in the characterization of the HLCs.

Reference	Method of stem cell differentiation			Differentiation efficiency % ALB +ve HLCs	PHENOTYPING: Phase I and II enzyme activity		
	Stem cell (cell line)	Culture format	Differentiation factors	% ALB +ve HLCs (assay method)	Enzyme (assay method)	% hPH comparator	Other comparators
Cai *et al.*, 2007(14)	hESC (H1, H9)	Monolayer, EB formation	AF V, AA, ITS, BMP2, FGF4, HGF, OSM, DEX	70 (ICC)	CYP2B6 (Fluorescence)	ND	hESC
Ek *et al.*, 2007(15)	hESC (SA002, SA002.5, SA167)	Monolayer	Proprietary differentiation medium, FGF2	ND	CYP1A1 (Fluorescence) CYP3A4 (Fluorescence)	0 0	– –
Söderdahl *et al.*, 2007(16)	hESC (SA001, SA002, SA002.5, AS034, SA121, and SA167)	Monolayer	Proprietary differentiation medium, bFGF	ND	GST (Fluorescence)	80	HepG2
Hay *et al.*, 2008(17); **Godoy et al., 2015**(11); **Cameron et al., 2015**(10)	hESC (H1, H9)	Monolayer	AA, Wnt3a	90 (ICC)	CYP 1A2 (LC-MS-MS) CYP1A2 (Luminescence) 100 CYP3A4 (Luminescence) 100	24	hESC
Shiraki *et al.*, 2008(18)	hESC (Khes-1)	Co-culture with M15 cell line	AA, BMP4, bFGF, HGF, DMSO, DEX, Ly294002	9 (ICC)	ND	–	–
Agarwal *et al.*,2008(19)	hESC (WA01, WA09)	Monolayer	AA, FGF4, HGF, BSA, OSM, DEX	67.4 (ICC)	ND	–	–
Moore *et al.*, 2009(20)	hESC (H1)	Monolayer, EB formation	AA, Wnt3a, HGF, OSM, DEX	72.8 (ICC)	CYP 1A2 (Fluorescence)	ND	hESC-derived HLCs in culture media of different components
Basma *et al.*, 2009(21)	hESC (H1)	Monolayer, EB formation	AA, FGF2, HGF, DMSO, DEX	55.5 (ICC)	CYP1A (Fluorescence) CYP3A (LC-MS-MS)	30 90	– –
Song *et al.*, 2009(22)	hESC (H1), hiPSC (hFb-derived 3U1, 3U2)	Monolayer	AF V, AA, ITS, BMP2, FGF4, OSM, DEX, KGF, B27	60 (ICC)	CYP2B6 (Fluorescence)	ND	hiPSC-derived versus hESC-derived HLCs
Duan *et al.*, 2010(23)	hESC (H9)	Monolayer	AA, sodium butyrate, BMP2, BMP4, FGF4, HGF DMSO, B27	75-90 (ICC, FACS)	CYP1A2 (LC-MS-MS) CYP2C9 (LC-MS-MS) CYP2D6 (LC-MS-MS) CYP3A4 (LC-MS-MS)	100 60 95 90	– – – –
Synnergren *et al.*, 2010(24)	hESC (SA002, SA167, SA461)	Monolayer	AA, ITS, FGF1, FGF2, BMP2, BMP4, HGF, OSM, DEX	ND	ND	–	–
Touboul *et al.*, 2010(25)	hESC (H9)	Monolayer	AA, BMP4,FGF2, FGF4, FGF10, HGF, EGF, retinoic acid, SB431542, Ly294002	ND	CYP3A (Bioluminescence)	ND	–
Brolén *et al.*, 2010(26)	hESC (SA001, SA002, SA002.5, SA167)	Monolayer	AA, BMP2, BMP4, FGF1, FGF2, HGF, OSM, DEX, Wnt3A	ND	CYP1A (LC-MS-MS) CYP2C (LC-MS-MS) CYP2A (LC-MS-MS)	ND	Spontaneously differentiated hESC-derived HLCs, HepG2
Ghodsizadeh *et al.*, 2010(27)	hiPSC (hFb-derived)	EB formation	AA, FGF2, HGF, DMSO, DEX	50 (FACS)	CYP2B6 (Fluorescence)	ND	hiPSC
Liu *et al.*, 2010(28)	hESC (WA01, WA09), hiPSC (hPH-derived)	Monolayer	AA, FGF4, HGF, OSM, DEX	ND	CYP1A2 (Bioluminescence) CYP3A4 (Bioluminescence)	ND	–
Si-Tayeb *et al.*, 2010(29)	hESC (H9), hiPSC (hFb-derived)	Monolayer	AA, BMP4, FGF2, OSM, B27	80 (FACS)	ND	–	–
Sullivan *et al.*, 2010(30)	hiPSC (hFb-derived)	Monolayer	AA, HGF, Wnt3A, DMSO, OSM, hydrocortisone, tryptose phosphate broth, B27	70-90 (ICC)	CYP1A2 (Bioluminescence) CYP3A4 (Bioluminescence)	ND	–

Rashid *et al.*, 2010(31)	hiPSC (hFb-derived)	Monolayer	AA, BMP4, FGF2, HGF, OSM, Ly294002, CHIR99021 (GSK-3 inhibitor)	83 (FACS)	CYP3A4 (Bioluminescence)	ND	hiPSC
Zhang *et al.*, 2011(32)	hESC (H9), hiPSC (hFb-derived)	Monolayer, EB formation	AA, BMP2, FGF4, HGF, KGF, OSM, DEX	60-80 (ICC, FACS)	CYP3A4 (Bioluminescence)	0.32	hESC-derived HLCs
Bone *et al.*, 2011(33)	hESC (Shef1, Shef3)	Monolayer	FGF4, HGF, OSM, DEX, 1 m (GSK-3 inhibitor)	ND	ND	–	–
Yildirimman *et al.*, 2011(34)	hESC (SA002)	Monolayer	Proprietary differentiation medium	ND	CYP1A2 (LC-MS-MS) CYP3A4 (LC-MS-MS) CYP2B6 (LC-MS-MS) CYP2C9 (LC-MS-MS) CYP2C19 (LC-MS-MS)	50 50 10 50 50	– – – – –
Chen *et al.*, 2012(35)	hESC (H9), hiPSC (hFb-derived, CFB46)	Monolayer	AA, ITS, HGF, Wnt3A, OSM, DMSO, DEX	ND	CYP3A4 (Bioluminescence)	100	hiPSC
Cayo *et al.*, 2012(36)	hiPSC (FH patient JD fibroblast-derived)	Monolayer	OCT4, SOX2, NANOG, LIN28	ND	ND	–	–
Schwartz *et al.*, 2012(37)	hiPSC (hFb-derived)	Monolayer	AA, BMP4, FGF2, HGF, OSM	80 (ICC)	ND	–	–
Takayama *et al.*, 2012(38)	hES (H9), hiPSC (hFb-derived, MCR5 & 201B7)	Monolayer	AA, SOX17, HEX, BMP4, FGF4, LacZ, HNF4α, HGF, OSM, DEX	ND	CYP3A4 (Fluorescence) CYP2C9 (Fluorescence) CYP1A2 (Fluorescence)	100 > 10 < 1	– – –
Choi *et al.*, 2013(39)	hiPSC (derived from AAT deficient patients)	Monolayer	B27, AA, FGF4, HGF, OSM, DEX	ND	CYP3A4 (Bioluminescence) CYP2D6 (Bioluminescence) CYP2C19 (Bioluminescence) CYP1A2 (Bioluminescence)	80 70 90 90	– – – –
Ramasamy *et al.*, 2013(40)	hESC (H1)	Monolayer & 3D culture in Algimatrix plate	AA, DMSO, HGF, OSM	ND	CYP3A4 (Bioluminescence)	ND	HepG2
Gieseck *et al.*, 2014(41)	hiPSC (hFb-derived)	Monolayer, 3D-single cell or Clump culture in RAFT system	AA, FGF2,BMP4, LY-294002, Hepatozyme-SFM	ND	CYP3A4 for 2D Day 35 (HPLC-MS) CYP3A4 for 3D Day 45 (Bioluminescence)	4 25	–
Jia *et al.*, 2014(42)	hiPSC (from urine cells of HA patient)	Monolayer, EB formation	AA, FGF4, BMP2, HGF, KGF, OSM, DEX	64 (FACS)	ND	–	–
Avior *et al.*, 2015(43)	hESC (I3)	Monolayer	AA, B27, Wnt3A, HGF, DMSO, DEX, OSM, FGF2, LCA, MK4	83 (FACS)	CYP3A4, 1A2 (Fluorescence) CYP2E1, 2C9 (Fluorescence)	30 8	HepG2, hESC without LCA/MK4
Chien et al., 2015(44)	hiPSC (from dental pulp stromal cells)	Co-culture with MEF, EB formation	AF V, AA, FGF4, BMP2, HGF, KFG, OSM, DEX, B27, miR122 (delivered by PU-PEI in CHC)	ND	ND	–	–

Abbreviations: AA, activin A; AF V, albumin fraction V; bFGF, human recombinant basic FGF; BMP, bone morphogenic protein; BSA, bovine serum albumin; CHC, carboxymethyl-hexanoyl chitosan; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FGF, fibroblast growth factor; GFP, green fluorescent protein; GSK, glycogen synthase kinase; Hepatozyme-SMF, hepatozyme serum free medium; HEX, hematopoietically-expressed homeobox protein; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; HLCs, hepatocyte-like cells; HGF, hepatocyte growth factor; HNF4alpha, hepatocyte nuclear factor 4 alpha; ICC, immunocytochemistry; ITS, insulin-transferrin-selenium; KGF, keratinocyte growth factor; LacZ, beta-D-glactosidase; Ly294002, phosphoinositide 3-kinase inhibitor; miR122, microRNA 122; ND, not determined; OCT, octamer-binding transcription factor; OSM, oncostatin M; PU-PEI, biodegradable polyurethane-graft-short-branch polyethylenimine; qRT-PCR, quantitative real time polymerase chain reaction; SB431542, inhibitor for activin receptor-like kinase receptors ALK5, ALK4 and ALK7; SOX, sex determining region Y-box; Wnt3a, wingless-type MMTV integration site family, member 3a



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