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Stem Cell–Derived Models to Improve Mechanistic Understanding and Prediction of Human Drug-Induced Liver Injury

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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 personalized toxicology to determine interindividual susceptibility to adverse drug reactions. However, the complexity of idiosyncratic drug-induced liver injury 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 drug-induced liver injury. 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. (Hepatology 2017;65:710-721).

Abbreviations: CYP, cytochrome P450; DILI, drug-induced liver injury; SC-HLC, stem cell–derived hepatocyte-like cell.

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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 are a significant clinical problem, resulting in considerable patient morbidity and mortality,[1] and thus represent a major financial burden on health care systems. Adverse drug reactions also represent a major challenge for the pharmaceutical industry, leading to attrition of drugs in development and the withdrawal of drugs postlicensing.[2] Among different forms of adverse drug reactions, the liver is particularly susceptible to drug toxicity; drug-induced liver injury (DILI) is the second highest cause of attrition and accounts for >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% (European Medicines Agency 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 United States the DILI Network (http://www.dilin.org/) and the International Serious Adverse Event Consortium (http://www.saeconsortium.org/) are attempting to address this issue. The clinical manifestation of DILI indicates that it is a multidimensional and multifaceted 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 costly and functionally variable (reflecting variation in

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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, while 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. While significant progress toward 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 hepatotoxicity and 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 (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 European Partnership for Alternative Approaches to Animal Testing/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 and 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 1 drug metabolism, which play a key role in the chemical functionalization and eventual elimination of drugs from the body but 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.[50]).

The recent studies outlined above[48,49] are particularly important as they suggest that modeling 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:

TABLE 1. Summary of Studies Post-2007 of HLC Derivation From Human Pluripotent Stem Cells: Note the Limited Number of Phase 1 and 2 Phenotyping Markers Generally Employed in the Characterization of the HLCs

	Method of stem cell differentiation			Differentiation Efficiency % ALB^+ HLCs	Phenotyping: Phase 1 and 2 Enzyme Activity		
Reference	Stem Cell (Cell Line)	Culture Format	Differentiation Factors	% ALB^+ HLCs (Assay Method)	Enzyme (Assay Method)	% hPH Comparator	Other Comparators
Cai et al.[17]	hESC (H1, H9)	Monolayer, EB formation	AF V, AA, ITS, BMP2, FGF4, HGF, OSM, DEX	70 (ICC)	CYP2B6 (fluorescence)	ND	hESC
Ek et al.[18]	hESC (SA002, SA002.5, SA167)	Monolayer	Proprietary differentiation medium, FGF2	ND	CYP1A1 (fluorescence) CYP3A4 (fluorescence)	0 0	— —
Soderdahl et al.[19]	hESC (SA001, SA002, SA002.5, AS034, SA121, and SA167)	Monolayer	Proprietary differentiation medium, bFGF	ND	GST (fluorescence)	80	HepG2
Hay et al.,[20] Godoy et al.,[14] Cameron et al.[13]	hESC (H1, H9)	Monolayer	AA, Wnt3a	90 (ICC)	CYP 1A2 (LC-MS-MS) CYP1A2 (luminescence) 100 CYP3A4 (luminescence) 100	24	hESC
Shiraki et al.[21]	hESC (Khes-1)	Coculture with M15 cell line	AA, BMP4, bFGF, HGF, DMSO, DEX, Ly294002	9 (ICC)	ND	—	—
Agarwal et al.[22]	hESC (WA01, WA09)	Monolayer	AA, FGF4, HGF, BSA, OSM, DEX	67.4 (ICC)	ND	—	—
Moore and Moghe[23]	hESC (H1)	Monolayer, EB formation	AA, Wnt3a, HGF, OSM, DEX	72.8 (ICC)	CYP 1A2 (fluorescence)	ND	hESC-derived HLCs in culturemedia of different components
Basma et al.[24]	hESC (H1)	Monolayer, EB formation	AA, FGF2, HGF, DMSO, DEX	55.5 (ICC)	CYP1A (fluorescence) CYP3A (LC-MS-MS)	30 90	— —
Song et al.[25]	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.[26]	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.[27]	hESC (SA002, SA167, SA461)	Monolayer	AA, ITS, FGF1, FGF2, BMP2, BMP4, HGF, OSM, DEX	ND	ND	—	—
Touboul et al.[28]	hESC (H9)	Monolayer	AA, BMP4, FGF2, FGF4, FGF10, HGF, EGF, retinoic acid, SB431542, Ly294002	ND	CYP3A (bioluminescence)	ND	—
Brolen et al.[29]	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

TABLE 1. *Continued*

Reference	Method of stem cell differentiation			Differentiation Efficiency % ALB+ HLCs	Phenotyping: Phase 1 and 2 Enzyme Activity		
	Stem Cell (Cell Line)	Culture Format	Differentiation Factors	% ALB+ HLCs (Assay Method)	Enzyme (Assay Method)	% hPH Comparator	Other Comparators
Ghodsizadeh et al.[30]	hiPSC (hFb-derived)	EB formation	AA, FGF2, HGF, DMSO, DEX	50 (FACS)	CYP2B6 (fluorescence)	ND	hiPSC
Liu et al.[31]	hESC (WA01, WA09),hiPSC (hPH-derived)	Monolayer	AA, FGF4, HGF, OSM, DEX	ND	CYP1A2 (bioluminescence) CYP3A4 (bioluminescence)	ND	—
Si-Tayeb et al.[32]	hESC (H9), hiPSC (hFb-derived)	Monolayer	AA, BMP4, FGF2, OSM, B27	80 (FACS)	ND	—	—
Sullivan et al.[33]	hiPSC (hFb-derived)	Monolayer	AA, HGF, Wnt3A, DMSO, OSM,hydrocortisone, tryptosephosphate broth, B27	70-90 (ICC)	CYP1A2 (bioluminescence) CYP3A4 (bioluminescence)	ND	—
Rashid et al.[34]	hiPSC (hFb-derived)	Monolayer	AA, BMP4, FGF2, HGF, OSM, Ly294002, CHIR99021 (GSK-3 inhibitor)	83 (FACS)	CYP3A4 (bioluminescence)	ND	hiPSC
Zhang et al.[35]	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.[36]	hESC (Shef1, Shef3)	Monolayer	FGF4, HGF, OSM, DEX, 1 m (GSK-3 inhibitor)	ND	ND	—	—
Yildirimman et al.[37]	hESC (SA002)	Monolayer	Proprietary differentiationmedium	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.[38]	hESC (H9), hiPSC (hFb-derived, CFB46)	Monolayer	AA, ITS, HGF, Wnt3A, OSM, DMSO, DEX	ND	CYP3A4 (bioluminescence)	100	hiPSC
Cayo et al.[39]	hiPSC (FH patient JD fibroblast-derived)	Monolayer	OCT4, SOX2, NANOG, LIN28	ND	ND	—	—
Schwartz et al.[40]	hiPSC (hFb-derived)	Monolayer	AA, BMP4, FGF2, HGF, OSM	80 (ICC)	ND	—	—
Takayama et al.[41]	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	— — —

TABLE 1. *Continued*

Reference	Method of stem cell differentiation			Differentiation Efficiency % ALB$^+$ HLCs	Phenotyping: Phase 1 and 2 Enzyme Activity		
	Stem Cell (Cell Line)	Culture Format	Differentiation Factors	% ALB$^+$ HLCs (Assay Method)	Enzyme (Assay Method)	% hPH Comparator	Other Comparators
Choi et al.[42]	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.[43]	hESC (H1)	Monolayer & 3D culture in Algimatrix plate	AA, DMSO, HGF, OSM	ND	CYP3A4 (bioluminescence)	ND	HepG2
Gieseck et al.[44]	hiPSC (hFb-derived)	Monolayer, 3D-single-cell or clump culturein 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.[45]	hiPSC (from urine cells of HA patient)	Monolayer, EB formation	AA, FGF4, BMP2, HGF, KGF, OSM, DEX	64 (FACS)	ND	—	—
Avior et al.[46]	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.[47]	hiPSC (from dental pulp stromal cells)	Coculture with MEF, EB formation	AF V, AA, FGF4, BMP2, HGF, KFG, OSM, DEX, B27, miR122 (delivered by PU-PEI in CHC)	ND	ND	—	—

Modified from tables 1 and 2 of Kia et al.[16]

Abbreviations: AA, activin A; AAT, alpha1-antitrypsin; AF V, albumin fraction V; ALB, albumin; bFGF, human recombinant basic FGF; BMP, bone morphogenic protein; BSA, bovine serum albumin; CHC, carboxymethyl-hexanoyl chitosan; 3D, three-dimensional; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EB, embryoid body; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FGF, fibroblast growth factor; FH, familial hypercholesterolemia; GSK, glycogen synthase kinase; GST, glutathione *S*-transferase; HA, hemophilia A; Hepatozyme-SFM, hepatozyme serum free medium; hESC, human embryonic stem cell; HEX, hematopoietically expressed homeobox protein; hFb, human fibroblast; HGF, hepatocyte growth factor; hiPSC, human induced pluripotent stem cell; HNF4α, hepatocyte nuclear factor 4 alpha; hPH, human primary hepatocyte; HPLC-MS, high-performance liquid chromatography–mass spectrometry; ICC, immunocytochemistry; ITS, insulin-transferrin-selenium; JD, initial of a cell donor adopted as the name of a cell line; KGF, keratinocyte growth factor; LacZ, beta-D-glactosidase; LC-MS-MS, liquid chromatography–tandem mass spectrometry; LCA, lithocholic acid; Ly294002, phosphoinositide 3-kinase inhibitor; MEF, mouse embryonic fibroblast; miR122, microRNA 122; MK4, menaquinone-4; ND, not determined; OCT, octamer-binding transcription factor; OSM, oncostatin M; PU-PEI, biodegradable polyurethane-graft-short-branch polyethylenimine; SB431542, inhibitor for activin receptor-like kinase receptors ALK5, ALK4, and ALK7; SOX, sex determining region Y-box; Wnt3a, wingless-type mouse mammary tumor virus integration site family, member 3a.

- 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 cocultivation 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 microbially derived secondary metabolites to which immature hepatocytes are likely to be exposed postpartum 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 that 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 micropatterned cocultures, 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 among the most challenging to mature, and we should 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 modeled 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 levels.[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 tumor models. Thus, the impact of drugs that cause simple single–ion channel or complex multichannel 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 interindividual variation in expression of xenobiotic metabolism and transporter proteins in addition to the chemistry of each drug.

While protocols to differentiate stem cells toward 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 toward progressing the complement 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

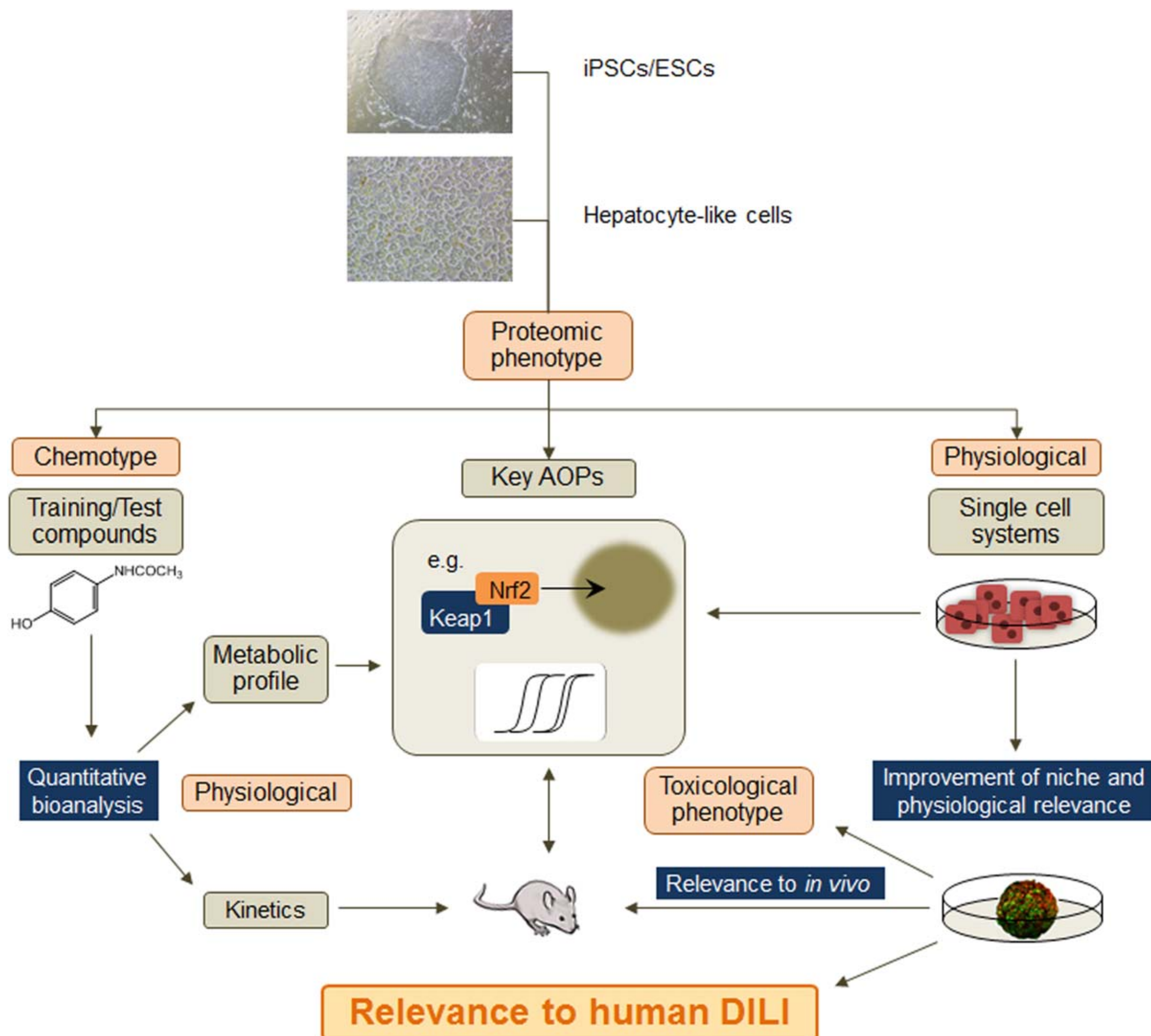

FIG. 1. Roadmap for producing stem cell-derived models to improve mechanistic understanding and prediction of human DILI. The physiological, pharmacological, and toxicological characterization of stem cell-derived hepatocytes is necessary before the cells can be fully utilized. This will include the use of toxicity/stress reporters and a small panel of well-defined chemicals, thereby defining the toxicological purpose for which each line is suitable. This will position the new cells within a screening toolbox that could be validated for drug/chemical safety evaluation. The use of iPSC lines with drug toxicity-relevant mutations and the use of CRISPR technology to edit genes involved in drug metabolism may also be important in this regard. Abbreviations: AOP, adverse outcome pathway; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

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 CYPs, some relevant phase 2 enzymes (such as the glutathione transferases and uridine diphosphoglucuronate-glucuronyl transferases), and some phase 3 proteins (influx and efflux transporters) are expressed at a set and reproducible percentage 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 Characterization

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 Fig. 1). Moreover, the phenotype of the HLCs must be as reproducible as possible, and they should be fully characterized, 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 characterize the cells as this would represent a broad visualization of the physiological phenotype of the cells. Similarity to freshly isolated hepatocytes/tissue can be established through proteomics and targeted multiple reaction monitoring–based mass spectrometric analysis of key proteins, such as CYPs, transporters, and intracellular signaling molecules, as well as determination of metabolic and cellular uptake profiles. Developments in mass spectrometric technologies mean that it is now possible to analyze small panels of proteins (for example, 10-20 transporters or P450s) using multiple reaction monitoring 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 article reporting only a 39% correlation between messenger RNA and protein at a global level,[65] measuring messenger RNA levels is not recommended for cell characterization purposes.

As part of a comprehensive assessment of HLC phenotype, recent developments in the field of hepatocyte-selective translatable biomarkers (e.g., microRNA 122[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 nuclear erythroid 2 p45-related factor 2, pregnane X receptor, and nuclear factor kappa B 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 systems to humans will be invaluable in this endeavor. 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 clustered regularly interspaced short palindromic repeats 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 characterization may be assisted by a better understanding of the mechanisms contributing to dedifferentiation 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 neighboring hepatocytes, disrupting their gap junctions and tight junctions, which are important for their phenotype, as well as their juxtaposed nonparenchymal 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 1 CYP capacity—that is most rapidly and profoundly depleted[71,75]; 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 reestablish a functional hepatocyte from stem cells is the investigation of the precise

cellular mechanisms underlying the dedifferentiation process that occurs in hepatocytes once they have been removed from the liver. While the factors driving dedifferentiation may not be identical to those that drive differentiation, it is likely that one or more pathways and processes uncovered through research into dedifferentiation 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, multidimensional 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 cells, will be valuable biological models to explore the physiological, pharmacological, and toxicological responses 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 characterize 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 overreliance 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 three-dimensional 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 functions of the cells, only once they have been properly phenotyped. There is little point in exposing HLCs to chemicals chosen as hepatotoxins in humans unless we fully characterize the cells.

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