REVIEW

Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening

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Received 23 December 2009; received in revised form 24 February 2010; accepted 25 February 2010

Abstract Hepatotoxicity is an enormous and increasing problem for the pharmaceutical industry. Early detection of problems during the drug discovery pathway is advantageous to minimize costs and improve patient safety. However, current cellular models are sub-optimal. This review addresses the potential use of pluripotent stem cells in the generation of hepatic cell lineages. It begins by highlighting the scale of the problem faced by the pharmaceutical industry, the precise nature of drug-induced liver injury and where in the drug discovery pathway the need for additional cell models arises. Current research is discussed, mainly for generating hepatocyte-like cells rather than other liver cell-types. In addition, an effort is made to identify where some of the major barriers remain in translating what is currently hypothesis-driven laboratory research into meaningful platform technologies for the pharmaceutical industry.

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Introduction

The clinical translation of human pluripotent stem cell (PSC) research into cell therapy for patients has rightly captured the public’s imagination for how healthcare might enjoy major advances in the 21st century. An alternative, yet similarly profound, opportunity for clinical benefit is the use of differentiated ‘non-clinical grade’ cells to screen the toxicity of putative new drugs in pharmaceutical development. In this review, we address the use of PSCs in pre-clinical hepatotoxicity screening. At present, most work in this area employs human embryonic stem cells (ESCs) differentiated towards hepatocytes, but this may be supplemented by the use of induced (i) PSCs. Previous articles in stem cell journals have focused on reviewing the relative merits of different differentiation protocols as has been described recently for the pancreatic beta cell (Van Hoof et al., 2009). Here, we update in this area, but also focus on the perspective of the pharmaceutical industry and their requirements for a pre-clinical model of toxicity testing. The considerations for screening new chemical entities (NCEs) during the drug discovery pathway stretch far beyond whether we can generate the perfect hepatocyte in the research laboratory.

The scale and importance of drug-induced liver injury

The potential value of applying human PSC research to hepatotoxicity screening of NCEs should not be understated. Unexpected toxicity and adverse drug events post-licensing are leading causes of compound attrition and product withdrawal, with up to 30% of compound failures occurring due to toxicity and clinical safety issues (Kola and Landis, 2004). In a series of high-profile cases where approved drugs have been withdrawn from the market, 50% were due to drug-related hepatotoxicity (Lee, 2003). A recent study found that 6.2% of all UK hospital admissions were related to adverse drug reactions (ADRs) (Pirmohamed et al., 2004). ADRs can be regarded as a significant burden on public health, with a 0.15% mortality rate and high economic costs associated with hospitalization of patients (Pirmohamed et al., 2004). One of the most common causes of toxicity-induced ADRs is drug-induced liver injury (DILI), however, the underlying mechanisms of tissue damage are complex, multi-dimensional, incompletely understood and not fully amenable to testing in cell culture systems (Goldring et al., 2006) (Fig. 1). Many drugs form reactive metabolites that can covalently bind to cellular macromolecules, and initiate and propagate liver injury (Usui et al., 2009). DILI caused through organelle dysfunction directly in human hepatocytes, for example mitochondrial (Labbe et al., 2008; Rachek et al., 2009) or endoplasmic reticular (ER) dysfunction (Lawless et al., 2008), is more amenable to testing in cell culture systems. Mitochondrial dysfunction is an important mechanism whereby pharmaceuticals can trigger serious liver injury through disruption of mitochondrial energy production and/or release of pro-apoptotic proteins into the cytoplasm, ultimately resulting in hepatocyte necrosis or apoptosis and cytolytic hepatitis (Labbe et al., 2008). Alternatively, mitochondrial dysfunction can lead to steatosis and steatohepatitis which may progress to cirrhosis (Labbe et al., 2008). Redox perturbation induced by drug exposure may lead to ER stress (Frosali et al., 2009). ER dysfunction may also be initiated by protein overload or mis-folding and lead to apoptosis. The ‘suicide’ option of apoptosis is the last line of defense for the hepatocyte. Prior to this, complex adaptations are possible (Fig. 2), which serve to reinforce the dynamic, complex phenotype of the hepatocyte in response to chemical stress.

Taken together, these multi-faceted risks associated with NCEs place high demands for effective and predictive toxicity screening models. The specific attributes of these models, including the potential use of human PSCs, also need to be tailored to the timing of their application during the drug discovery pathway.

The drug discovery pathway - the need for better cell models

In recent years, the pharmaceutical industry has faced considerable challenges related to the reduced productivity of NCEs, increased production costs, long research and development timelines, and high attrition rates. Factoring in these
issues, the cost of bringing a drug to market is thought to be USD $0.9-1.7 billion (Kola, 2008). One study, published in 2004, analyzed the rate of success for registering products compared to the number of ‘first-in-man’ studies over a decade (1991-2000) for the 10 largest US and European pharmaceutical companies (Kola and Landis, 2004). On average, the success rate was just 11% with the vast majority of failures occurring in late Phase III trials or at registration. These late failures are associated with enormous financial costs. The US Food and Drugs Administration (FDA) felt that stagnation in the drug discovery pathway was so serious that it commissioned a report in 2004 to analyze pipeline problems (http://www.fda.gov/oc/initiatives/criticalpath/whitepaper.htm).

Traditionally, the bottleneck in the discovery pathway was thought to be the identification of hit compounds based on, amongst other criteria, assay performance, analyses of structure-activity relationships, potency and dose response effects (Fig. 3). However, as in silico technologies for high-throughput screening have improved, attrition rates have not, indicating that the bottleneck has shifted downstream to the phase of ‘lead optimization’ (Bleicher et al., 2003). Lead optimization aims to improve the attributes of a compound as a candidate in 2004 to analyze pipeline problems.

Figure 1  Liver injury. The liver is the primary organ for metabolic biotransformation of xenobiotics, including drugs, and is consequently a frequent target for a variety of hepatotoxic insults. Chemically-mediated toxicity can affect any cell-type and mimic any naturally occurring disease process. Frequently, the initial toxic insult is followed by involvement of the immune system with a resulting inflammatory reaction.

Figure 2  The multi-tiered defence response against chemically-induced toxicity within the liver. Diagram to show the range of defense responses adopted by the liver in reaction to increasing severity of chemical stress. Initial defence is provided through constitutive expression of antioxidant proteins and low molecular weight scavengers (e.g. glutathione), as well as phase II drug metabolizing enzymes and transporters. Subsequently these defences are bolstered through a transcriptionally induced up-regulation of defence proteins, principally orchestrated through the factor Nrf2. Finally, the cell is scheduled to undergo apoptotic self-destruction as an alternative to necrotic cell death, which carries with it the added risk of an inflammatory response.
drug (or, potentially, develop a pro-drug that would only be converted to its active form after administration) by altering chemical structure, as well as considering absorption, distribution, metabolism and elimination properties. Critically, lead optimization also involves assessment of toxicity in a range of tests. Animal studies, both in vitro and in vivo, are a useful tool in the drug development process and a number of species, including rodents, dog and monkey, are commonly used for testing potential toxicity of NCEs before the first dose is administered in man. However, species differences in the activities of orthologous drug metabolizing enzymes, which may produce hepatotoxic metabolites in a species-specific manner mean that extrapolation to man from such models must be undertaken with caution (Martignoni et al., 2006). Between synthetic in vitro assays of cytochrome P450 (CYP) activity and animal studies, the FDA report identified a pressing need for better in vitro cell models to broaden understanding of the biochemical and genetic basis of DILI, and to develop predictive liver toxicity biomarkers for hepatotoxicity screening; hence, the interest in human PSC research. The goal is two-fold and interlinked: to demonstrate the safety and effectiveness of potential lead compounds in faster time-frames, with more certainty, at lower costs; and to identify unfavorable toxicity profiles during lead optimization thereby reducing attrition rates in late-stage development.

**Cellular models of hepatotoxicity—what is the standard for pluripotent stem cell research?**

**Requirements for effective hepatotoxicity screening**

The liver is composed of hepatocytes, making up 70% of liver mass, and other cell-types such as Kupffer cells, stellate cells, biliary epithelial cells, endothelial cells and, potentially, infiltrating immune cells. The organ has a precise tissue architecture (Fig. 4), which, along with the characteristics of the composite cell population, contributes to the toxicity or resolution of a particular chemical insult (Liu et al., 2004; Imaeda et al., 2009) (Fig. 1). Therefore, cell models for hepatotoxicity should ideally incorporate all these components and faithfully represent the myriad functions a liver performs in vivo. As a priority, the model’s capacity to metabolize xenobiotics is paramount, which requires expression of CYP enzymes that are inducible by various liver-enriched nuclear receptors and transcription factors (Goldring et al., 2004; Itoh et al., 1997; Itoh et al., 1995). CYPs are of particular importance in the generation of hepatotoxicants (Goldring et al., 2006; Antoine et al., 2008), however, not all hepatotoxicity is metabolism-mediated (Iwanaga et al., 2007). Amongst the first consequences of chemical and oxidative insult is NFκB-induced cytokine release (Bowie and O’Neill, 2000). Another liver function essential to the resolution of toxic assault is the ability to process and eliminate any reactive intermediates through conjugation (Bock et al., 1987) or transport (Szakacs et al., 2008). Therefore, in addition to expressing hepatic enzymes (Table 1), model cells must have commensurate pools of the cognate factors and co-factors.

**Human hepatocellular carcinoma cell lines**

The availability and ease-of-culture of hepatocellular carcinoma-derived cell lines have made their application to toxicity studies an attractive proposition, but in reality their utility is hindered by incomplete and abnormal metabolic and transport pathways (Castell et al., 2006). The major CYPs may be poorly expressed or absent and other isoforms associated with tumour...
cells can be over-expressed, making extrapolation of their in vitro metabolism profiles to in vivo liver function potentially misleading. Nevertheless, they can be useful in the study of particular enzyme isoforms following transient or stable transfection (Goldring et al., 2006; Aoyama et al., 2009). Recently there have been reports of a novel cell line (HepaRG) which can be expanded in an undifferentiated state and on reaching confluence can be differentiated into both hepatocyte- and biliary-like cells (Guillouzo et al., 2007; Parent et al., 2004). The former cells express a good complement of mRNAs encoding drug metabolizing proteins, demonstrate CYP3A4, CYP1A2 and UDP-glucuronosyltransferase activity comparable to primary human hepatocytes (Kanebratt and Andersson, 2008) and have been suggested as suitable for chronic toxicity and genotoxic studies (Josse et al., 2008). However, the clonal nature makes the cell line representative of only one genotype.

Primary human hepatocyte culture

Isolated primary human hepatocytes are the current ‘gold-standard’ in vitro model as they express the entire complement of hepatocyte drug metabolizing enzymes and transporters (Hewitt et al., 2007). Dissociation and culturing purified hepatocytes in monolayer obviously compromises model complexity compared to the native organ, however, overlaying extracellular matrix enhances maintenance of the differentiated hepatocyte phenotype, including cell polarity, for days or weeks (Lecluyse, 2001; Moghe et al., 1996). Co-culture of hepatocytes with epithelial cells (Rogiers and Vercruysse, 1993), stellate cells (Riccaltion-Banks et al., 2003) and pancreatic cells (Kaufmann et al., 1999) has also been investigated as a step towards designing systems with enhanced liver complexity. Despite this, there are many issues that remain problematic with the use of primary human hepatocytes. Supply is scarce as relatively small numbers can be isolated from tissue derived from planned surgical resections. Large numbers of hepatocytes may occasionally become available from whole organs deemed unsuitable for transplantation, but by nature this tissue is often of poorer quality and unpredictable in supply. Such issues have stimulated research on cryopreservation (Terry and Hughes, 2009), which has made progress, although there is inevitable variation between batches (Rojmans et al., 2004). As a toxicity screening platform for the pharmaceutical industry, batch-to-batch consistency and ability to ship cryopreserved cells without loss of function are important. Even in optimised culture systems, hepatocytes still lose the differentiated phenotype that is essential for meaningful study of drug metabolism and toxicity (Elaut et al., 2006). Dedifferentiation includes losing the ability of certain CYPs and phase II genes to be induced by reference compounds (Park et al., 1996), in part due to the activation of stress signaling pathways and the generation of nitric oxide (Sidhu et al., 2004; Lopez-Garcia, 1998; Rodriguez-Ariza and Paine, 1999; Vernia et al., 2001; Aitken et al., 2008; Olsavsky et al., 2007; Page et al., 2007).

Nitrile levels are inversely correlated to CYP levels and activity; the latter decline with increased nitrosylation, haem loss and degradation (Lopez-Garcia, 1998; Vernia et al., 2001; Aitken et al., 2008). Nitric oxide-producing stimuli also reduce the levels of liver enriched nuclear factors and nuclear receptors (Fang et al., 2004; Beigneux et al., 2002).

Differential pharmacodynamic and toxic responses in vivo and in vitro arise from the great diversity of metabolic phenotypes in human populations. These may be genetic, environmental, dietary, occupational, disease-related, or drug-induced and explain, in large part, the differences
### Table 1: Characteristics for defining human PSC-derived hepatocyte-like cells.

<table>
<thead>
<tr>
<th>A</th>
<th>Liver cell type</th>
<th>Gene</th>
<th>% ESTs</th>
<th>Conflicting expression in:</th>
<th>Discriminatory genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal hepatoblast</td>
<td>AFP</td>
<td>21</td>
<td>Fetal heart, extraembryonic endoderm</td>
<td>MLC2V (fetal heart), MUC1 (fetal lung)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALB</td>
<td>63</td>
<td>Mature hepatocyte, fetal lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TDO2</td>
<td>58</td>
<td>Fetal heart, fetal lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature hepatocyte</td>
<td>OATP2</td>
<td>89</td>
<td>Highly specific</td>
<td>MUC1 (fetal and mature lung), AFP (fetal liver, EEE), MLC2V (fetal heart), HAND2 (ciliary ganglion)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALB</td>
<td>63</td>
<td>Fetal heart, fetal lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAT</td>
<td>93</td>
<td>Mature lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TDO2</td>
<td>58</td>
<td>Fetal heart, fetal lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAT</td>
<td>85</td>
<td>Ciliary ganglion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biliary cell/ cholangiocyte</td>
<td>KRT17</td>
<td>&lt;0.1</td>
<td>Bronchial epithelium</td>
<td>KRT14 (bronchial epithelium), ACPP (prostate), MUC1 (fetal lung), PDX1 (fetal pancreas), SIX2 (fetal kidney)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KRT19</td>
<td>3</td>
<td>Many other cell-types</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HNF1B</td>
<td>8</td>
<td>Fetal kidney, fetal pancreas</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B. Functional assessment

<table>
<thead>
<tr>
<th>Method overview</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen storage</td>
<td>Periodic acid–Schiff staining</td>
<td>Useful but also occurs in muscle and undifferentiated ESCs</td>
</tr>
<tr>
<td>Urea production</td>
<td>Measurement of synthesis and excretion by detection in cell-free conditioned medium</td>
<td>Specific hepatocyte function</td>
</tr>
<tr>
<td>Albumin secretion</td>
<td>Measurement of protein in cell-free conditioned medium</td>
<td>Relatively insensitive test—other functional properties are lost before albumin secretion</td>
</tr>
<tr>
<td>Low density lipoprotein (LDL) uptake and metabolism</td>
<td>Detection of labelled intracellular uptake and degradation</td>
<td>Useful but also occurs in vascular endothelial cells and macrophages</td>
</tr>
<tr>
<td>Indocyanine green (ICG)</td>
<td>Hepatocytes in culture take up and secrete ICG dye over 6 h</td>
<td>Difficult to apply to cultured cells due to lack of functional bile excretion system in vitro</td>
</tr>
</tbody>
</table>

### C. CYP Substrates

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate</th>
<th>Inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>theophylline, polycyclic aromatic hydrocarbons (PAH)</td>
<td>PAH, e.g., benzopyrene</td>
</tr>
<tr>
<td>1A2</td>
<td>caffeine, melatonin, warfarin</td>
<td>rifampicin, phenobarbital</td>
</tr>
<tr>
<td>2A6</td>
<td>coumarin, nicotine, quinoline, valproic acid, paracetamol</td>
<td>rifampicin, phenobarbital, dexamethasone</td>
</tr>
<tr>
<td>2B6</td>
<td>nictoine, bupropion, cyclophosphamide, efavirenz</td>
<td>clotrimazole, rifampicin, phenobarbital</td>
</tr>
<tr>
<td>2C8</td>
<td>ibuprofen, paclitaxel, cerivastatin</td>
<td>rifampicin, carbamazepine</td>
</tr>
<tr>
<td>2C9</td>
<td>warfarin, phenytoin, linoleic acid, rosiglitazone, tolbutamide</td>
<td>rifampicin, carbamazepine, hyperforin</td>
</tr>
<tr>
<td>2C19</td>
<td>amitriptyline, dione, timolol, flecainide, dextromethorphan</td>
<td>rifampicin, hyperforin, prednisolone</td>
</tr>
<tr>
<td>2D6</td>
<td>debrisoquine, dione, timolol, flecainide, dextromethorphan</td>
<td>piperidines, dexamethasone, rifampicin</td>
</tr>
<tr>
<td>2E1</td>
<td>ethanol, paracetamol, halothane, toluene</td>
<td>acetone, ethanol</td>
</tr>
<tr>
<td>3A4</td>
<td>testosterone; glutathione transferases – 1-chloro-2,4-dinitrobenzene, UDPGT – 4-methylumbelliferone; OATP – ICG</td>
<td>rifampicin, phenobarbital, hyperforin, carbamazepine</td>
</tr>
</tbody>
</table>

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A. Genes expressed in human fetal hepatoblast, mature hepatocyte and biliary epithelial cells to discriminate these cell-types from others that might arise during PSC differentiation. ‘% ESTs’ refers to the percentage of expressed sequence tags derived from liver in the Unigene database; high values indicate expression that is restricted to liver. No gene is uniquely expressed in liver, however, the expression of ‘discriminatory genes’ should not be detected in fetal hepatoblast, mature hepatocyte or biliary epithelial cell respectively. B. Functional analyses that can be undertaken to assess human PSC-derived hepatocyte-like cells in comparison to freshly isolated human primary hepatocytes. C. CYP enzymes expressed in mature hepatocytes along with their metabolic substrates and reference compounds known to induce their expression and / or activity.
demonstrated by pools of hepatocytes isolated from different donors (Ponsoda et al., 2001). This genotype-dependent expression of important CYPs, such as CYP3A isoforms, CYP4A2, CYP2A6, CYP2B6, CYP2D6, and several CYP2C isoforms, would cause highly variable toxicity readouts for NCEs. Ideally, this genotypic diversity needs to be reflected in banks of primary hepatocytes or equivalent depositories of PSC-derived cells (Ingelman-Sundberg et al., 2007). Not all variability in gene expression is due to sequence differences; the expression of microRNAs (miRNA) and gene methylation are additional control mechanisms that may play a role in the adverse down-regulation of CYP expression in isolated and primary cultured cells, and cell lines (Ingelman-Sundberg et al., 2007; Gomez and Ingelman-Sundberg, 2009). Methylation of CpG islands in the gene promoters of several of the most important CYPs have been identified, the transcripts from which may also be regulated by miRNAs interacting with the 3’ UTR, as is the case for CYP3A4 and CYP2B6 (Ingelman-Sundberg et al., 2007).

Despite these problems, primary cultures of adult human hepatocytes remain the current gold standard in human metabolism and hepatotoxicity studies. They play a pivotal role in drug discovery and development (Soars et al., 2009; Soars et al., 2007) and they set the standard against which PSC-differentiated progeny need to be judged.

Generating liver cell-types from human pluripotent stem cells

Theoretically, PSC-derived liver cells have a number of advantages over primary adult hepatocytes for drug testing. They can potentially give rise to all of the composite cells of the adult human liver, thus could provide a complexity for toxicity screening that begins to approximate the intact organ (Fig. 4) (Itskovitz-Eldor et al., 2000). They could also generate liver cells at different stages of maturity; to assess the effects of drugs at different stages of liver development. The genetic diversity that underlies the major differences in metabolizing genotype can also be represented by access to different genotyped ESC or iPSC lines (Taylor et al., 2005; Nakatsuji et al., 2008). iPSC technology has the potential to generate liver cell-types with particular disease phenotypes, providing disease-specific cell models for therapeutic drug development and testing. PSCs prior to differentiation are more available and can be cryopreserved with retention of phenotype more readily than primary hepatocytes (Baxter et al., 2009; David et al., 2009). A ready supply of liver cells from a particular ESC or iPSC line would also improve reproducibility during screening by avoiding the batch to batch variability of primary adult hepatocytes. All these attributes have stimulated interest that human PSCs can be applied to drug toxicity screening during lead compound optimization and improve understanding of the biochemical pathways involved in human metabolism. To anticipate use of PSC-derived hepatocyte-like cells to replace all current pharmaceutical practices would be naïve, but their integration alongside other types of screening tests is feasible; for instance, in helping to minimise laboratory animal usage. The major challenge is to develop robust platforms for the differentiation of the desired cell-types from PSCs on a scale and practicality that improves on current practice by the pharmaceutical industry. The following sections update on progress in this area and highlight where gaps remain.

Development of the liver

In vitro protocols for differentiating PSCs to hepatocytes draw heavily on knowledge of how the liver normally develops and matures during embryogenesis and the fetal period (Fig. 5). The three germ layers of the embryo are formed during gastrulation which begins at E6.5 in mice (Kubo et al., 2004). During this period, epiblast cells fated to become definitive endoderm undergo epithelial-mesenchymal transition (EMT) at the anterior primitive streak under the influence of Wnt and high levels of Nodal signalling (Kubo et al., 2004; Murry and Keller, 2008; Nakanishi et al., 2009). Folding turns the flat sheet of definitive endoderm, regulated by the transcription factors SOX17, GATA4 and FOXA2, into a gut tube (Wells and Melton, 1999). Hepatogenesis begins at E8.5–9 from the ventral foregut endoderm in progenitor cells that harbour an equal capacity to form the ventral bud of the pancreas (Deutsch et al., 2001; Kamiya et al., 1999). Hepatic specification of these bipotential cells is induced by dual signalling from two adjacent mesodermal sources: fibrolast growth factor (FGF) 1, FGF2 and FGF8 from the neighbouring cardiac mesoderm; and bone morphogenetic protein (BMP) 2 and BMP4 from the septum transversum (Jung et al., 1999; Chung et al., 2006; Rossi et al., 2001). Once specified, the hepatoblasts proliferate under autocrine signals and ones from surrounding angioblasts, and invade the stroma of the septum transversum (Jung et al., 1999; Lemaigre and Zaret, 2004; Matsumoto et al., 2001). They are bipotent, expressing markers for fetal hepatocytes [e.g. hepatocyte nuclear factor (HNF) 4a, HNF6, alpha-fetoprotein (AFP) and albumin (ALB), and biliary epithelium (e.g. cytokeratin (KRT) 17 and KRT19)] (Lavon and Benvenisty, 2005; Tanimizu and Miyajima, 2004) (Fig. 5). At E10, haematopoietic cells colonize the nascent liver bud. This induces Notch signalling, which acts to differentiate the bipotential hepatoblasts by promoting or inhibiting differentiation into either biliary duct epithelium or hepatocytes respectively (Lavon and Benvenisty, 2005; Tanimizu and Miyajima, 2004). The haematopoietic cells also produce the cytokine, oncostatin M (OSM), which, in combination with glucocorticoid, acts as a liver maturation factor (Kinoshita et al., 1999). Hepatocyte growth factor (HGF) from the septum transversum and surrounding nonparenchymal liver cells also plays important roles over a long time course by promoting hepatocyte proliferation, survival and maturation (Zaret, 2000; Kamiya et al., 2001). Complete hepatocyte maturation only occurs after birth (Kamiya et al., 2001).

Differentiating pluripotent stem cells towards hepatocyte-like cells

At present, efforts to generate hepatocyte-like cells have been predominantly from ESCs rather than iPSCs, although the first advances with the latter cell-types have recently been published (Song et al., 2009; Sullivan et al., 2009). So far, methods developed using ESCs have also been found broadly applicable using iPSCs (Song et al., 2009; Gareth et al., 2010). Various strategies have been applied, most of which aim to simulate in vivo liver development in the in vitro setting.
In vitro simulation of in vivo liver development

Differentiation of liver cell-types can be divided into three steps: definitive endoderm differentiation, hepatocyte progenitor specification and hepatocyte maturation (Fig. 5). In addition, it is possible to consider three components, which collectively form an evolving niche for differentiation: soluble growth factors; supporting cell-types; and extracellular matrix (ECM).

**Soluble growth factors.** The first protocol to describe differentiation of ESCs towards hepatocytes used embryoid body (EB) formation to induce initial differentiation (Hamazaki et al., 2001). EB formation by ESC aggregation, potentially with subsequent plating onto adhesive substrates (such as Matrigel or collagen) as EB outgrowth cultures (Itskovitz-Eldor et al., 2000), serves as an in vitro mimic of gastrulation and subsequent lineage specification. However, the differentiation is random (Kubo et al., 2004; Pei et al., 2009; Soto-Gutierrez et al., 2006; Momose et al., 2009). To prioritise hepatocyte differentiation, cultures have been treated with exogenous factors and the resulting hepatocyte-like cells characterised by their gene expression profile and characteristics (Momose et al., 2009; Duan et al., 2007) (Table 2). Subsequent protocols have aimed to avoid the inherent random differentiation in EB-based protocols by the direct induction of ESCs cultured in monolayer. As a mimic for the in vivo Nodal and Wnt signalling at the anterior end of the primitive streak, initial definitive endoderm differentiation of ESCs has been routinely induced by Wnt3a and high concentrations of Activin A (commonly 100 ng/ml) (Nakanishi et al., 2009; Cai et al., 2007; Zhao et al., 2009). Conversely, phosphatidylinositol 3-kinase (PI3K) is known to block endoderm differentiation from ESCs (D’Amour et al., 2005), such that low serum concentrations or specific PI3K inhibitors (e.g. LY294002) have been used alongside Activin A (Mclean et al., 2007; Shiraki et al., 2008).

Following definitive endoderm formation, characterised by expression of GATA4, FOXA2 and SOX17 (D’Amour et al., 2005), hepatocyte specification has been mimicked by the addition of FGF and BMP family members (Cai et al., 2007; Gouon-Evans et al., 2006), to mirror the secretion of these factors from the cardiac mesoderm and septum transversum respectively (Chung et al., 2008; Neta Lavon, 2005). In vitro, FGF2 or FGF4 and BMP2 or BMP4 have been added to specify hepatocyte progenitors (Cai et al., 2007; Zhao et al., 2009; Shiraki et al., 2008; Gouon-Evans et al., 2006).

For final maturation of hepatocyte progenitors, specialised hepatocyte growth medium has been devised (Jasmund et al., 2007) with a number of added maturation factors. These factors typically include HGF, shown to support the expansion of fetal hepatocytes (Kamiya et al., 2001; OSM, known to induce differentiation and maturation of fetal hepatocytes (Kamiya et al., 1999; Kinoshita et al., 1999) and dexamethasone, a potent synthetic glucocorticoid, which induces the activity of hepatocyte enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT) and members of the CYP family (Neta Lavon, 2005; Duanmu et al., 2002).
<table>
<thead>
<tr>
<th>Ref</th>
<th>Differentiation method</th>
<th>Characterization</th>
<th>Liver-associated markers</th>
<th>Functional assessment</th>
<th>Comments / limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EB Monolayer Co-culture</td>
<td>ECM Factors added</td>
<td>RT-PCR</td>
<td>ICC</td>
<td>WB</td>
</tr>
<tr>
<td>(Rambhatla et al., 2003)</td>
<td>+ + M DMSO, Na Butyrate, HGF</td>
<td>+ + - - + + + + + - + + - + + - + - + -</td>
<td>AFP not detected in Na butyrate-treated cultures.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Lavon et al., 2004)</td>
<td>+ - FGF1, FGF2, BMP4, HGF</td>
<td>+ + - + + - - - - - - - - - -</td>
<td>Each growth factor added individually, not altogether. Very simple differentiation protocol.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Cai et al., 2007)</td>
<td>- + MEF AA, FGF4, BMP2 HGF, OSM, DEX</td>
<td>+ + - - + + + + + + + + + +</td>
<td>CYP activity levels not compared to adult hepatocyte control. Very low levels of liver integration in vivo.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Duan et al., 2007)</td>
<td>+ + C DEX</td>
<td>+ + - + + + + + + + + + +</td>
<td>Method relies on spontaneous hepatocyte ESC differentiation and microdissection for hepatocyte enrichment. Variable TAT expression.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ek et al., 2007)</td>
<td>- + MEF FGF2</td>
<td>+ - + - - - - - - + - - - - - - - - -</td>
<td>No CYP activity despite detection of CYP transcript and protein.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Hay et al., 2007)</td>
<td>- + M DMSO, HGF, OSM</td>
<td>+ + + - + + + - + + + + + + + - + -</td>
<td>Low efficiency: only foci of hepatocyte-like cells, only 10% of which were positive for ICG. No positive control for CYP activity assay.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Condition</td>
<td>Matrix</td>
<td>Factors</td>
<td>Outcomes</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
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<td>------------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>(Agarwal et al., 2008)</td>
<td>- +</td>
<td>C</td>
<td>AA, FGF4, HGF, OSM, DEX</td>
<td>No data on CYP enzyme activity levels. Low/sporadic liver integration and differentiation.</td>
<td></td>
</tr>
<tr>
<td>(Baharvand et al., 2008)</td>
<td>- +</td>
<td>M</td>
<td>FGF2, Noggin, aFGF, FGF4, HGF, DEX, OSM</td>
<td>No expression of G6P, marker of hepatocyte maturation. Adult primary hepatocytes not used as control in functional assays</td>
<td></td>
</tr>
<tr>
<td>(Chiao et al., 2008)</td>
<td>+ -</td>
<td>FGF1</td>
<td>+ + - + + - + - + - - - - - - -</td>
<td>AFP positive cells selected by reporter gene expression. Very simple differentiation protocol.</td>
<td></td>
</tr>
<tr>
<td>(Hay et al., 2008b)</td>
<td>- +</td>
<td>M</td>
<td>AA, Na Butyrate, DMSO, HGF, OSM</td>
<td>Low CYP protein + activity levels compared to adult hepatocyte control.</td>
<td></td>
</tr>
<tr>
<td>(Ishii et al., 2008)</td>
<td>+ +</td>
<td>C, L</td>
<td>HGF, BMP4, FGF4, AA all-trans retinoic acid</td>
<td>Aim of study to assess the affect of extracellular matrices on hepatic differentiation. No functional tests.</td>
<td></td>
</tr>
<tr>
<td>(Jasmund et al., 2007)</td>
<td>- +</td>
<td>M15 cells</td>
<td>AA, FGF2, LY294002 HGF, DEX</td>
<td>Low efficiency: 9% albumin positive cells after 40 days differentiation. Transplantation of ASGR sorted population enriched for liver markers. Recipients developed adenocarcinoma.</td>
<td></td>
</tr>
<tr>
<td>(Basma et al., 2009)</td>
<td>+ +</td>
<td>M</td>
<td>AA, FGF2, HGF DMSO, DEX</td>
<td>Transplantation of ASGR sorted population enriched for liver markers. Recipients developed adenocarcinoma.</td>
<td></td>
</tr>
</tbody>
</table>

(continued on next page)
<table>
<thead>
<tr>
<th>Ref</th>
<th>Differentiation method</th>
<th>Characterization markers</th>
<th>Liver-associated markers</th>
<th>Functional assessment</th>
<th>Comments / limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Moore and Moghe, 2009)</td>
<td>EB Monolayer Co-culture</td>
<td>ECM Factors added</td>
<td>RT-PCR, ICC, WB, FACS</td>
<td>AFP, AAT, HNF4, TAT</td>
<td>CYP activity levels only compared to undifferentiated ESC no positive adult primary hepatocyte control.</td>
</tr>
<tr>
<td>(Pei et al., 2009)</td>
<td>- + MEF</td>
<td>DEX, OSM, HGF, AA, WNT3A</td>
<td>+ + - + + + - + - + -</td>
<td>- + + - - - + + + -</td>
<td>No data on CYP activity levels. Limited panel of markers used to characterize hepatocytes.</td>
</tr>
<tr>
<td>(Song et al., 2009)</td>
<td>- - fetal liver cell line</td>
<td>AA, HGF, OSM, DEX</td>
<td>+ + - - + - - - + + + -</td>
<td>- - - - + + + + -</td>
<td>Very low levels of albumin secretion, urea synthesis, albumin secretion and CYP3A4 activity in comparison to adult hepatocytes.</td>
</tr>
<tr>
<td>(Zhao et al., 2009)</td>
<td>- + STO</td>
<td>AA, BMP2, FGF4, HGF, KGF, OSM, DEX</td>
<td>+ + - + + + + + + + + + +</td>
<td>+ + + + + + + + -</td>
<td>Albumin secretion and CYP activity levels not compared to adult hepatocyte control.</td>
</tr>
</tbody>
</table>

In addition to abbreviations used throughout the article text, those used in the Table are: AA, activin A; C, collagen; DEX, dexamethasone; iMEFs, inactivated mouse embryonic fibroblasts; ICC, immunocytochemistry; KGF, keratinocyte growth factor; L, laminin; M, Matrigel; Na butyrate, sodium butyrate; STO, a specific mouse embryonic fibroblast cell line; VEGF, vascular endothelial growth factor; WB, western blot.
Co-culture with supporting cell-types. Similar to the recognised benefits of culturing PSCs on inactivated mouse embryonic feeder cells (MEFs) (Thomson et al., 1998), differentiating cells in vitro toward hepatocytes can benefit from contact with other cells (Fair et al., 2003). During development hepatoblasts interact with both the cardiac and septum transversum mesoderm; in teratomas, hepatocyte-like cells have been observed to differentiate alongside cardiac cells (Lavon et al., 2004). ESCs co-cultured with both embryonic chick mesoderm (Fair et al., 2003) and a mesoderm-derived cell line (M15) (Shiraki et al., 2008) demonstrated enhanced hepatocyte differentiation. Other examples include the use of human non-parenchymal liver cell lines, which have been shown to support mature hepatocyte culture by the production of hepatocyte mitogens (Kang et al., 2004). Similarly, Zhao and colleagues found that ESC-derived hepatic progenitor cells could be expanded upon murine embryonic stromal cells (Zhao et al., 2009). Alternatively, feeder cell lines have been genetically modified to produce specific growth factors, such as FGF2, in order to enhance hepatocyte differentiation (Pei et al., 2009).

Extracellular matrix. It is difficult to attribute how much of the benefit from co-culture comes from the provision of ECM by the supporting cell-type rather than from cellular contact. Several ECM substrates have proven beneficial. In vivo, the liver bud expands into the collagen-rich stroma of the septum transversum (Hamazaki et al., 2001; Agarwal et al., 2008). Two substrates regularly used for in vitro differentiation of hepatocyte-like cells are collagen and, arguably better (Ishii et al., 2008), Matrigel, a proprietary matrix from BD Biosciences, one of the constituents of which is collagen (Kleinman and Martin, 2005). A natural extension of this work has been the development of 3D scaffolds, which potentially improve mimicry of the in vivo developmental microenvironment. Collagen scaffolds have supported the generation of functional hepatocyte-like cells differentiated both directly from ESCs and via EBs (Baharvand et al., 2006; Imamura et al., 2004).

Epigenetic manipulation of hepatocyte-like differentiation from embryonic stem cells

The first protocol to differentiate hepatocyte-like cells from human ESCs utilised selection of hepatocyte progenitor cells by the histone deacetylase inhibitor, sodium butyrate, which caused massive cell death of presumptive non-hepatic cells and was thought to help maintain CYP activity (Rambhatla et al., 2003). More recently, these cells have been expanded and matured by addition of HGF and OSM (Hay et al., 2008b). DMSO can similarly affect histone acetylation and promote hepatocyte-like differentiation (Hay et al., 2007). Mechanistically, it remains unclear why such widespread epigenetic modulation can be effective leading to these agents being termed unspecific (Sharma et al., 2006; Zhou et al., 2007).

Lineage tracing to enrich for hepatocyte-like differentiation from embryonic stem cells

Despite the improvements made to protocols in differentiating hepatocyte-like cells from PSCs, cultures remain heterogeneous. Therefore, many strategies have been built in the use of PSCs capable of expressing stably transfected fluorescent reporter genes (e.g. enhanced green fluorescent protein (EGFP)) under the control of a liver-specific promoter or enhancer, commonly from the AFP, ALB or α1-antitrypsin (AAT, officially known as SERPINA10) genes (Duan et al., 2007; Lavon et al., 2004; Ishii et al., 2008). This has allowed real-time tracking of differentiating cultures, tracing of transplanted cells in vivo and the selection of appropriately differentiated cells by FACS (Basma et al., 2009). AFP expression reflects hepatocyte maturity: it is abundantly expressed in the fetal liver, but is extinguished in adult hepatocytes and only re-expressed as a marker of regeneration or hepatocellular carcinoma (Chen et al., 1997; Lopez, 2005). In keeping with this profile, hepatocyte-like cells selected by AFP reporter gene expression exhibited no mature hepatocyte function beyond ALB secretion (Duan et al., 2007; Ishii et al., 2008; Chiao et al., 2008). In contrast, AAT is expressed in hepatocyte progenitors and mature hepatocytes. Enrichment of hepatocyte-like cells differentiated from ESCs and EB outgrowth cultures by selecting for lentiviral expression of an AAT-EGFP transgene exhibited liver-specific functions such as CYP activity, and indocyanine green dye (ICG) uptake and secretion, as well as hepatic gene and protein expression (Duan et al., 2007). In this study, following random differentiation of plated EBs up to 14% of cells expressed EGFP and, surprisingly, exhibited CYP1A2 activity comparable to primary human hepatocytes (Duan et al., 2007).

Characterisation and analysis of hepatocyte-like cells derived from embryonic stem cells

A summary of characterisation from different studies is provided in Table 2. The initial formation of definitive endoderm has been characterised by the transient expression of the primitive streak marker Brachyury, and a combination of FOXA2, GATA4 and SOX17; (D’Amour et al., 2005) (Fig. 5). The subsequent generation of hepatoblasts has been defined by ALB and AFP expression (Soto-Gutierrez et al., 2007). More mature hepatocyte-like cells, suggested by characteristic epithelial morphology, have been described according to immunostaining for a combination of ALB along with CK18, and transcription factors, such as HNF4a, CEBPα and CEBPβ, in the absence of AFP (Duan et al., 2007; Monaghan et al., 1993; Baharvand et al., 2008). Similar status has been inferred by expression of a range of liver-specific enzymes such as CYPs, TAT and AAT (Duan et al., 2007; Cai et al., 2007; Zhao et al., 2009). In some studies, functional characteristics have been obtained. During repair following liver injury, transplanted ESC-derived hepatocyte-like cells carried a selective advantage (Shafrirz and Dabeva, 2002), and have been shown to integrate and differentiate into hepatocytes in host liver (Gouon-Evans et al., 2006; Agarwal et al., 2008), secrete ALB and AAT (Duan et al., 2007; Basma et al., 2009), improve hepatic function and increase survival rates (Soto-Gutierrez et al., 2006; Ishii et al., 2007). Similarly, their use in bioartificial liver devices improved short-term (5-day) survival rates of rats with fulminant hepatic failure (Cho et al., 2008).

Unmet demands and future challenges for hepatocyte-like cells generated from pluripotent stem cells

Despite the initial progress, there remain several shortcomings and barriers to the use of cells currently derived from human PSCs as a toxicity screening platform during drug discovery.
Standardizing initial characterisation of hepatocyte-like cells

At present the characterisation of hepatocyte-like cells generated from human PSCs has tended to reflect the hypothesis-driven experimentation of individual, independent research laboratories. Most protocols have described the appearance of cells with typical hepatocyte morphology, however, there has been a general lack of agreed standards, equivalent for instance to the ‘minimum information about a microarray experiment (MIAME)’ parameters required to deposit expression microarray data in public repositories (Brazma, 2009; Brazma et al., 2001). In redress, Sancho-Bru et al. have made very helpful recommendations for the characterisation of stem cell derived hepatocyte-like cells (Sancho-Bru et al., 2009), highlighting minimal criteria by which different protocols can be compared. In Table 1A, we also provide a profile of gene expression, reliant on both positive and negative markers, attempting to discriminate hepatocyte-like and biliary-like cells from other cell-types. The major problem is that genes expressed during hepatocyte differentiation are also commonly expressed in other organs and tissues (e.g. AFP in extra-embryonic endoderm (Gualdi et al., 1996), and FOXA2 in other endodermal and ectodermal tissues (Neta Lavon, 2005)). Analysing the origin of human expressed sequence tags (ESTs) in the Unigene database reinforces this scale of this problem: only 21% of ESTs for human AFP originated from liver; most came from human fetal heart libraries making the inclusion of ‘negative control’ genes, such as MLC2V, particularly important (Table 1A). This issue can compromise differentiation strategies reliant on reporter genes expressed under the control of regulatory elements from a single ‘hepatocyte-specific’ gene (Soto-Gutiérrez et al., 2006; Duan et al., 2007; Ishii et al., 2007). Where AFP-positive cells have been selected via reporter gene expression, other hepatocyte markers including TAT and tryptophan 2,3-dioxygenase (TDO2) have been weakly detected and albumin has only been expressed in a minority of cells, suggestive of significant extra-embryonic differentiation (Ishii et al., 2008).

When it comes to mature hepatocytes, commonly used markers can also be expressed in other tissues, for example HNF4α in the kidney, pancreas, testis and intestine (Drewes et al., 1996). However, highly restricted gene expression profiles are available for mature hepatocytes. Although CYPs are surprisingly widely expressed, all bar one human EST for the organic anion transporter OATP2 (officially known as SLC10A1) and AAT (officially known as SERPINA10), arose from liver (the others were from breast and fetal heart, respectively). When coupled to the expression of other markers, like TDO2 and TAT, it is possible to clearly define the human hepatocyte by the expression of surprisingly few genes. The same does not appear true of the biliary epithelial cell, where greater reliance on the absence of negative markers becomes important (Table 1A).

Functional attributes of hepatocyte-like cells for drug toxicity platforms

Whereas thorough gene expression profiling may indicate a cell’s lineage and ‘baseline’ phenotype, it is in vitro mimicry of dynamic hepatocyte function that is paramount for effective NCE toxicity screening during drug discovery. Alongside the widely appreciated importance of inducible CYP activity, this also includes albumin secretion, urea synthesis, glycogen storage, and uptake and excretion of ICG (Duan et al., 2007; Agarwal et al., 2008; Hay et al., 2007; Cho et al., 2008) (Table 1B-C). However, as with gene expression profiling, some of these attributes in isolation are not hepatocyte-specific; for instance, undifferentiated ESCs, as well as myocytes, can also accumulate intracellular glycogen deposits (Johkura et al., 2004).

Given the importance of CYP activity for drug inactivation, pro-drug activation or the generation of toxic metabolites, assessment of these enzymes in PSC-derived hepatocyte-like cells is critical and, as yet, incomplete. No studies to date have demonstrated adequate CYP induction in PSC-derived hepatocyte-like cells in response to known reference compounds (Tables 1C and 2). Whereas some studies of human PSC-derived hepatocyte-like cells have detected CYP transcripts, such as CYP7A1 and CYP3A4, by RT-PCR (Duan et al., 2007; Hay et al., 2008a), actual enzyme concentrations have rarely been quantified and/or activity is often not equated to the gold standard of freshly isolated human primary hepatocytes (Hay et al., 2007); although providing preliminary clues, less helpful comparisons have been made to undifferentiated ESCs or hepatocyte cell lines with limited CYP activity (e.g. HepG2) (Hay et al., 2008a; Novik et al., 2008; Wilkening et al., 2003; Hewitt and Hewitt, 2004; Rodrá-Guez-Antona et al., 2002; Ek et al., 2007). Certainly, very few protocols have described CYP activity that is near-comparable to that of adult hepatocytes (Duan et al., 2007) (with care to ensure no loss of enzyme activities in the latter during in vitro culture (Jasmund et al., 2007).

Purity of hepatocyte-like differentiation versus the value of multi-cellular complexity

A mixed population comprising only a small proportion of hepatocyte-like cells could be responsible for the low levels of enzyme activity that have been reported to date from differentiated PSC cultures (Hay et al., 2008b). Indeed, hepatocyte differentiation, determined by FACS analysis for more than one marker (Gouon-Evans et al., 2006), seems inefficient (between 10-30%) (Hay et al., 2007; Basma et al., 2009) making subsequent enrichment steps necessary. Basma et al. described cell selection via detection of asialoglycoprotein receptor (ASGPR) yielding cells with levels of CYP1A1/CYP1A2, AAT, complement factor VII (CFVII) activity and urea comparable to primary hepatocytes (Basma et al., 2009).

The opposite perspective from trying to isolate pure hepatocyte-like cells is to actively encourage protocols that incorporate other liver cell-types thereby trying to create a more complete in vitro mimic of physiological liver function. Progress has been made in reporting biliary-like epithelial differentiation by modifying methodology after the specification of hepatoblasts (Zhao et al., 2009). Other important cell-types to consider include hepatic stellate cells as a model for agents capable of inducing liver fibrosis.
Phenotypic stability and scale-up of hepatocyte-like cells derived from pluripotent stem cells

Primary hepatocytes undergo limited expansion ex vivo before they lose their mature cell phenotype even with manipulations such as collagen or Matrigel overlay (Elaut et al., 2006; Jasmund et al., 2007). Therefore, it seems reasonable to predict that PSC-derived hepatocyte-like cells will undergo similar dedifferentiation. This poses questions as to when to harvest a population of hepatocyte-like cells differentiated from PSCs: if the mature phenotype is only transient, attaining pure mature populations seems unlikely unless PSC differentiation is synchronised. A more likely endpoint would be heterogeneous PSC-derived cells at different stages of hepatocyte-like maturity. Synchronising differentiation is technically difficult if not biologically impossible; even undifferentiated ESC cultures are heterogeneous for critical transcription factors such as Nanog (Chambers et al., 2007). Furthermore, colonies of ESCs are not uniform and often consist of a peripheral margin of spontaneously differentiated cells (Johkura et al., 2004).

Scale-up is mandatory for practical use by the pharmaceutical industry. The expansion of the differentiated, mature hepatocytes is technically difficult but the expansion of hepatic progenitor cells could be more achievable. ESC-derived bipotential hepatic progenitors have been expanded for more than 100 days whilst maintaining their phenotype in terms of gene expression and the capacity to differentiate into hepatocytes and biliary epithelial cells (Zhao et al., 2009). However, the ability of the former cells to induce CYP activity in response to reference compounds was not compared to human adult hepatocytes.

Using hepatocyte-like cells derived from pluripotent stem cells for in vivo drug testing

In vitro liver cell models have their limitations in terms of preserving the activity and maturity of the hepatocyte phenotype; an alternative would be an in vivo, artificial human liver for testing NCEs (Yoshizato and Tateno, 2009). Several models of mouse chimeras with humanized livers have been described. For instance, the chimeric urokinase-type plasminogen activator (uPA)+/−/severe combined immunodeficient (SCID) transgenic mouse line in which the liver can be over 90% repopulated with human hepatocytes (Katoh et al., 2008). These humanized mice express human phase I and phase II enzymes and exhibit humanized drug metabolism (Katoh et al., 2008). As such they are a powerful model for pre-clinical drug development. Potentially, the uPA+/−/SCID mouse liver could be repopulated with PSC-derived hepatocyte-like cells to exhibit a metabolic capacity reflective of the particular genotype of the human cells.

In vivo transplantation of PSC-derived liver cells might also solve the problem of generating fully functional, mature hepatocytes in vitro. The engraftment of ESC-derived pancreatic endoderm cells, resembling immature, fetal pancreatic tissue into SCID mice facilitated their final differentiation and maturation. Results showed that three months post-transplantation the implant contained glucose-responsive insulin-secreting cells that were functionally very similar to transplanted adult human islets (Kroon et al., 2008). Similarly, transplantation of PSC-derived hepatocyte-like cells might promote their full maturation. To date, such experiments have resulted in low and transient integration of the human cells, even when they have a proliferative advantage over the host cells (Duan et al., 2007; Agarwal et al., 2008; Ishii et al., 2007), however, in vivo maturation might be achievable by implanting cells on a bioartificial liver device as reported by Soto-Gutierrez et al. (Soto-Gutierrez et al., 2006). Furthermore, although there is potential for a high degree of humanization of mouse models there would still remain the underlying species differences either from the remaining murine hepatocyte complement or from other murine-specific effects.

Ensuring the necessary genetic diversity

There is increasing evidence that different ESC lines exhibit differing propensities for differentiation into specific lineages (Osafune et al., 2008), including hepatocytes (Ex et al., 2007). As such, a direct comparison between hepatocyte differentiation methods is often difficult because different protocols have been developed using different ESC lines. However, with better differentiation protocols and future iPSC lines, it seems feasible to anticipate panels of hepatocyte-like cells representing the different genotypes that underlie varying drug metabolising phenotypes. This is similar in principle to HLA matching of potential ESC derived transplants, where up to 150 selected different PSC lines have been estimated to be necessary (Taylor et al., 2005). The use of iPSC-derived hepatocytes would also avoid the ethical issues for some from the use of ESC, however the complete replacement of ESC with iPSC must be carefully considered. For instance, iPSC lines that have been reprogrammed using retroviruses may exhibit abnormal gene expression depending on the location of the retroviral insertion sites. There is also evidence that iPSC exhibit residual somatic gene expression from their originating cell type (Ghosh et al., 2010). As such, in our opinion, ESCs currently remain the archetypal PSC from which to generate liver cell models.

Other commercial considerations and practicalities

Whereas the above details a number of major barriers that will need surmounting for human PSCs to be applied as drug toxicity platforms, other issues will influence potential take-up by the pharmaceutical industry. These matters include robustness, ease of use, avoidance of inactivated feeder layers, cryopreservation, survival after shipping and batch-to-batch consistency.

Summary

There has now been almost a dozen years of fruitful human ESC research (Thomson et al., 1998; D’Amour et al., 2006; Lamba et al., 2009; Kim et al., 2007; Baker et al., 2007). Rapid progress has occurred during this short time, largely in the arena of discovery science. It is relatively easy to predict that the next ten years will begin translation of this basic knowledge into clinical applications for human benefit. The potential for better hepatotoxicity platforms to test NCEs is exciting; the challenge now is to close the gap between hypothesis-driven
research and pharmaceutical industry requirements, moving from differentiating the first hepatocyte-like cells in a dish to generating high-throughput platforms and standard operating procedures that are widely attainable in a cost-effective, efficacious manner.

Acknowledgments

The authors are part of the Stem Cells for Safer Medicine (www.SC4SM.org) consortium, from which they have received funding, and the MRC Centre in Drug Safety Science held at the Universities of Liverpool and Manchester. The authors would also like to acknowledge the support of EPSRC, BBSRC, the Wellcome Trust and the Manchester NIH Biomedical Research Centre.

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Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening


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