Review

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The potential of induced pluripotent stem cell derived hepatocytes

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Summary

Orthotopic liver transplantation remains the only curative treatment for liver disease. However, the number of patients who die while on the waiting list (15%) has increased in recent years as a result of severe organ shortages; furthermore the incidence of liver disease is increasing worldwide. Clinical trials involving hepatocyte transplantation have provided encouraging results. However, transplanted cell function appears to often decline after several months, necessitating liver transplantation. The precise aetiology of the loss of cell function is not clear, but poor engraftment and immune-mediated loss appear to be important factors. Also, primary human hepatocytes (PHH) are not readily available, de-differentiate, and die rapidly in culture. Hepatocytes are available from other sources, such as tumour-derived human hepatocyte cell lines and immortalised human hepatocyte cell lines or porcine hepatocytes. However, all these cells suffer from various limitations such as reduced or differences in functions or risk of zoonotic infections. Due to their significant potential, one possible inexhaustible source of hepatocytes is through the directed differentiation of human induced pluripotent stem cells (hiPSCs). This review will discuss the potential applications and existing limitations of hiPSC-derived hepatocytes in regenerative medicine, drug screening, *in vitro* disease modelling and bioartificial livers.

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Introduction

The demand for a stable source of functional hepatocytes is continuously growing, in addition to the requirement for viable organs for transplantation [1]. The former is due to the increased use of hepatocytes in various applications including drug screening, disease modelling and cell based therapies.

From the clinical perspective, transplantation of hepatocytes may represent an alternative to orthotopic liver transplantation (OLT) for specific life-threatening liver diseases. The aim of hepatocyte transplantation (HT) in such diseases, where the liver is normal, is to partially replace the missing function without the need to replace the whole organ. In this circumstance, the transplanted hepatocytes have both the time and the opportunity to engraft. The types of liver diseases can be broadly grouped into three categories:

(i) *Chronic liver diseases due to metabolic genetic disorders*. Metabolic diseases account for 26% of the indications for paediatric orthotopic liver transplantation in Europe (ELTR: www.eltr.org/). The successful replacement of deficient liver functions by transplantation of healthy hepatocytes has been reported in patients with Crigler-Najjar syndrome due to UGT1 enzyme deficiency, familial hypercholesterolemia due to low density lipoprotein receptor (LDLR) deficiency, and with urea cycle enzyme deficiencies [1]; the main cause to date is children with urea cycle defects. These diseases represent the major clinical application of cell therapy. It has been recently reported that inherited metabolic disorders could also be the

most common group of disorders that cause acute liver failure (ALF) in paediatrics. However, the use of HT in these cases has not yet been discussed [2].

(ii) *ALF that does not damage normal tissue architecture but is associated with direct injury and loss of hepatocytes.* HT in patients with ALF aims to restore liver function for a period of time, bridging to OLT or until the native liver regenerates. To provide sufficient function, the number of cells required for the treatment of ALF needs to be higher than for metabolic disorders. The severity of liver dysfunction requires that the transplanted hepatocytes function immediately, but it remains unclear if hepatocyte engraftment can occur on a clinically relevant time scale. Although restoration of liver function with full recovery of patients has been reported, in most published cases HT did not affect the clinical outcome of these patients. Encapsulation of hepatocytes in alginate microbeads which allows the cells to be transplanted intraperitoneally with the advantage of avoiding immunosuppression or extracorporeal artificial liver devices could offer a "bridge" allowing endogenous liver to recover spontaneously [1,3].

(iii) *Chronic liver failure with accompanying cirrhosis characterized by expansion of extracellular matrix, loss of fenestrae, widespread tissue damage and scar-based remodelling.* HT has also been used in patients with chronic liver disease with variable outcomes probably due to the presence of fibrosis, which makes it difficult for cells to cross the sinusoidal barrier and engraft. To date ectopic sites of cell transplantation are being considered [4].

The current benchmark cell type for all the applications envisioned for hepatocytes is freshly isolated primary human hepatocytes (PHHs). Isolation of hepatocytes competes with organs for OLT. Therefore, current sources of tissue for hepatocyte isolation are mainly adult organs that are unsuitable for OLT, or "healthy" livers, that have often already undergone aggressive therapies, or tissue surrounding excised tumours, and are often of a marginal quality. These cells are in limited supply, suffer from batch to batch variability and exhibit declining function in culture. In an effort to overcome the limited supply of PHHs, various groups have investigated the ability to cryopreserve PHH. Despite progression in these processes, cryopreservation still has detrimental effects on the viability and metabolic function of hepatocytes [5,6]. PHHs also lose their ability to proliferate when isolated from the normal *in vivo* microenvironment.

The problems with PHH quality and availability are not limited to cell therapy applications. Indeed, rapid loss of functionality in culture and batch to batch viability pose significant challenges for their use in drug screening and disease modelling, as long term analysis and reproducibility are mandatory for the generation of accurate datasets. Absence of proliferation and reduced functions of thawed hepatocytes hinder their potential for cell based therapies as large amounts of hepatocytes are required per treatment ($1–3 \times 10^8$ cells/kg body weight). The cells should also be readily available when needed for emergency treatment of ALF, and for planned and repeated treatment of liver-based metabolic disorders. The other existing sources of hepatocytes are tumour-derived human hepatocyte cell lines, immortalised human hepatocyte cell lines, adult stem/progenitor cells, foetal progenitors [7]. Each of these sources also suffer from one or a combination of various limitations such as reduced metabolic function, short term survival in culture, limited availability and proliferation ability [5,6,8], which hampers their use in the different applications. The HepaRG cell line represents an improvement over previous cell line models in terms of hepatocyte functions, particularly in CYP function and nuclear receptor pathways [9]. However, this cell line is derived from a tumour, resulting in reduced sensitivity to toxic insult. As for all hepatoma cell lines, it is also limited to a single genotype available for toxicity investigations, and thus is not representative of a broad patient population. Xenogeneic primary hepatocytes, in addition to the zoonotic risk by porcine endogenous retroviruses (PERVs) [10], also differ phenotypically from PHHs, resulting in different susceptibility to pathogens, drug metabolism and transporter activity, and are therefore regarded as unsuitable for many *in vitro* applications.

A large amount of research is being carried out focusing on the generation of a stable source of viable and functional hepatocytes. One possible inexhaustible source of hepatocytes is human induced pluripotent stem cells (hiPSCs) which have the capacity to indefinitely self-renew and to differentiate into all cell types, excluding extra-embryonic tissues. Compared to human embryonic stem cells (hESCs), hiPSCs have less ethical concerns and can be generated from unlimited sources with varying genetic backgrounds [11]. Therefore, hiPSC-derived hepatocytes (hiPSC-HEPs) hold significant potential as promising tools for drug discovery, cell therapy, *in vitro* disease modelling and bioengineered livers (Fig. 1). In addition to describing the use of hiPSC-HEPs in the mentioned applications, the review will also cover the current developments in the direct differentiation of hiPSCs into functional hepatocytes. The limits of using

Abbreviations: (h)iPSC(s), (human) induced pluripotent stem cell(s); hESC(s), human embryonic stem cell(s); hiPSC-HEP(s), hiPSC-derived hepatocyte(s); A1AT, α1-Antitrypsin; WD, Wilson's disease; FH, familial hypercholesterolemia; FTA, familial transthyretin amyloidosis; GSD1a, glycogen storage disease type 1a; ER, endoplasmic reticulum; LDLR, Low Density Lipoprotein Receptor; Dil-LDL, 3, 3′-dioctadecylindocarbocyanine-low density lipoprotein; HCV, hepatitis C virus; GMP, good manufacturing practices; BMP4, bone morphogenic protein 4; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HNF4α, hepatocyte nuclear factor 4α; CYP, cytochrome p450; CHIR99021, GSK-3 Inhibitor; hPSC-HEP(s), human pluripotent stem cell derived hepatocyte (s); HLA, human leukocyte antigen; TALENs, transcription activator-like effector nucleases; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9; uPA, urokinase-type plasminogen activator; 2D or 3D cultures, 2 or 3 dimensional cultures; LSEC(s), liver sinusoidal endothelial cell(s); BAL, bio-artificial liver; iPSC-HE, hiPSC-derived hepatic endoderm; HUVECs, human umbilical vein endothelial cells; iPSC-LB, iPSC-derived liver buds; iPSC-MH, "monolayer hepatocytes" conventionally 2D differentiated from iPSCs by contrast with iPSC-LB; ECM, extracellular matrix; UPD, uniparental disomy; CGH-array, comparative genomic hybridization-array; SNP, single nucleotide polymorphism; CNV(s), Copy Number Variation(s); PCSK9, Proprotein Convertase Subtilin/Kexin 9.

Key point

Hepatocyte transplantation now appears as a credible and promising bridge before, or even as an alternative to orthotopic liver transplantation in many life-threatening liver diseases but is hampered by cell shortage.

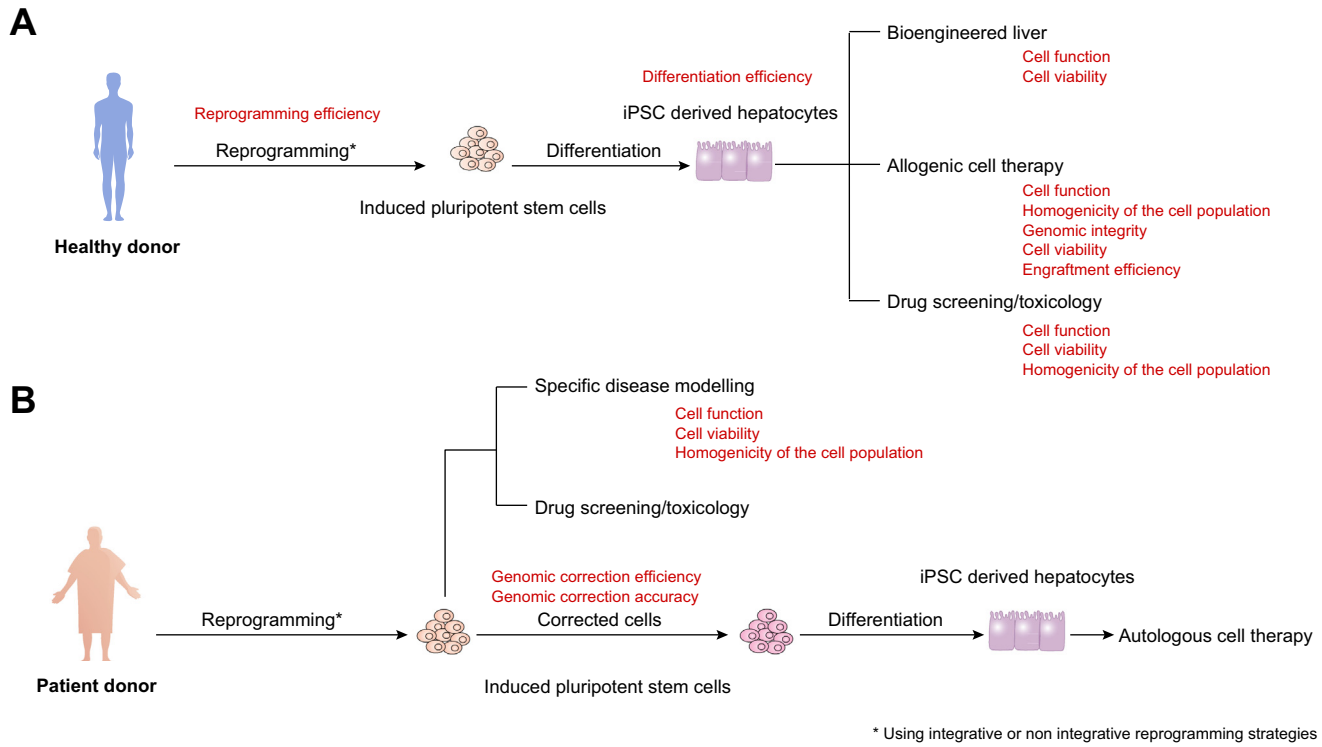

Fig. 1. Applications of hiPSC-derived Hepatocytes and their limitations (red) isolated from health donors (A) and patients (B).

hiPSC-HEPs in terms of function and safety will also be discussed, with a specific regard to the importance of the genomic integrity of the cells.

hiPSC generation and differentiation

Advancements in the generation of hiPSCs

Emerging techniques improving hiPSC differentiation efficiency would be superfluous without a common effort to optimise the upstream process, i.e., hiPSC generation. Remarkable advances have been demonstrated since the work of Yamanaka and colleagues in 2007, defining the proof of principle of reprogramming human fibroblasts into pluripotent stem cells using a retroviral vector [12]. The first efforts were focused on developing alternatives to integrative viral-mediated strategies, and as a result hiPSCs can currently be routinely generated using excisable viral vectors [13], RNA-Sendai virus vector [14], episomal plasmids transfections [15], miRNA transfections [16] or mRNA transfections [17]. Interestingly, from mouse fibroblasts, scientists generated iPSCs using only chemical compounds [18], but this has yet to be reproduced using human cells. Individual reprogramming strategies carry their own advantages and drawbacks, which also depend on the starting cell type. The first hiPSC lines were generated using fibroblasts cultured from a skin biopsy. However, it subsequently appeared that additional cell types were also susceptible to reprogramming such as keratinocytes [19], blood cells (lymphocytes [20], endothelial-colony forming cells [21], mesenchymal stem cells [22] etc.). Interestingly, cells collected from urine were successfully reprogrammed [23] even using non-integrative strategies [24]. Finally, various studies have confirmed the feasibility of generating and cultivating hiPSCs in stirred suspension bioreactors [25,26], driving this technology one step closer to applications that require large number of cells such as bioartificial livers (BAL) and high throughput drug screening.

Advancements in the generation of hepatocyte-like cells

In addition to the advancements in generating hiPSCs, there have been significant developments with regards to defining the protocols for the differentiation of hepatocytes from human pluripotent stem cells (hPSCs). The most important characteristics are acknowledged to be the ability to ensure the generation of functional and viable cells by recapitulating the developmental pathways while adhering to GMP compatible conditions. Three main factors need to be considered to ensure the successful differentiation of pluripotent stem cells into hepatocytes, these include: the extracellular matrix, additional cell-cell interactions and the media and supplements such as growth factors and cytokines. To date, the majority of differentiation protocols

have utilised undefined matrices such Matrigel® [27,28], however defined matrices that are easier to transpose in GMP conditions, such as collagen [29], laminin [30], fibronectin [31], vitronectin [32] or a specific combination of each [33], are currently being adopted. Various studies defining the media, growth factors and cytokines required to generate hepatocytes from pluripotent stem cells have already been performed. Despite the differences, all the published protocols recapitulate the major developmental steps leading to liver organogenesis with initial differentiation of the hiPSCs into definitive then hepatic-specified endoderm, followed by the formation of hepatic progenitors (hepatoblasts) and finally differentiation into functional and viable hepatocyte-like cells. WNT signalling has been shown to be implicated at earlier stages during human liver development [34]. The roles that Activin A and WNT3 signalling play during the establishment of the early primitive streak, which ultimately leads to endoderm specification, has been identified [35–37]. Various groups [27,36] target these two pathways in order to generate definitive endoderm (DE) cells. The WNT signalling pathway is also known to stabilize the Smad2/3 transcriptional network during the endoderm differentiation and to activate FOXA2 via interaction between β-catenin and SOX17, a marker of endoderm [38]. This pathway has other functions such as PI3K inhibition, necessary to allow Activin A to induce differentiation rather than the maintenance of the pluripotency network [39]. However, in serum-free conditions, WNT3A alone is ineffective in suppressing pluripotency genes expression and needs synergetic function of Activin A to promote DE differentiation [40]. Once accompanied with Activin A, DE differentiation efficiency induced by WNT3A or BMP4 is identical.

On the other hand, the specification of the primitive streak to mesendoderm then mesoderm or endoderm was shown to be dependent on Activin/Nodal signalling [41]. However, this pathway is also involved in maintaining the pluripotency network and is thus unable to promote this process. Activin A efficiently specifies DE from human embryonic stem cells only when phosphatidylinositol 3-kinase signalling is suppressed [42]. Thus other groups [43–45] activate the BMP4 and FGF2 pathways as well as Activin A signalling to drive endoderm specification using LY294002, an inhibitor of PI3kinase. The derivation of the latter protocol stems from prior developmental studies that highlight the importance of the FGF/BMP [46] pathways in hepatic induction and specification. BMP2 and 4 operate in accordance with FGF signalling, as these cytokines are secreted during liver development by the adjacent cardiac mesoderm and septum transversum mesenchyme, respectively. They function to encourage hepatic competence and specification of the primitive endoderm via GATA4 activation, a GATA zinc finger transcription factor [47–49]. BMP4 and FGF2 are considered as initiators of the differentiation into DE [50,51]. However, high concentrations of BMP4 in combination with FGF2 can drive differentiation to mesoderm from the mesendoderm stage [50]. Thus a combination of the WNT and Activin/Nodal signalling pathways seems to be the most suitable to generate DE as it reduces the risk of inducing mesoderm. However, the high concentration needed to boost DE differentiation and cost considerations may also be taken into consideration for the protocol choice.

There is a more defined consensus with regard to the factors needed for hepatic maturation due to the recapitulation of normal liver development. Developmental studies identified hepatocyte growth factor (HGF) and Oncostatin M (OSM) as promoters of hepatocyte differentiation. HGF is secreted by an array of cell types present in the liver including the septum transversum mesenchyme, endothelial cells. OSM is an IL-6 related cytokine produced by hematopoietic cells. As such, the majority of protocols have now agreed that both factors are the key components required to drive hepatocyte differentiation together with dexamethasone, which are known to induce expression of mature hepatic specific genes [27,36,43,45,52–54]. In such cases, hepatocyte-like cells can be successfully generated, expressing hepatocyte markers such as albumin, hepatocyte nuclear factor 4α (HNF4α) and some cytochrome p450s (3A4 and 7A1) whilst exhibiting various hepatocyte functions, such as urea and serum albumin production and selective drug metabolism [43,55,56]. Although the majority of current developments are focused on defining the media and matrices using GMP compatible products, the production of safe and fully functional human hepatocytes from hiPSCs for clinical applications remains to be achieved. In order to address this issue, a number of groups are employing small molecules and chemical compounds. In 2013, a study by Shan *et al.* isolated a number of various chemical compounds that promoted hepatic differentiation of iPSCs [57]. Taken further, Siller *et al.* recently derived a protocol that solely uses small molecules to drive the differentiation of pluripotent stem cells to hepatocytes [58]. CHIR99021 was used to form DE, supported by other studies [37,43,59,60], followed by treatment with DMSO, dexamethasone, hydrocortisone-21-hemisuccinate and Ile-(6) aminohexanoic amide (dihexa) to drive hepatic maturation. The resulting hepatocytes exhibited pentagonal morphology and expressed hepatic specific markers such as albumin, AFP and alpha 1 antitrypsin (A1AT). Functional analysis of the cells revealed

comparable levels of serum protein production and CYP450 activity when compared to hiPSC-HEPs derived using growth factor based protocols. Provided that a deeper analysis of these cells demonstrates the efficacy, reproducibility and safety of these small molecules, this study brings the field one step closer to a scalable, reliable and efficient hepatocyte differentiation protocol.

To date, no gold standard method for assessing the hPSC-derived hepatocytes (hPSC-HEPs) exist and the current assessments lack the standardization of phenotypic and functional characterization of hPSC-HEPs. As a result, this has made comparisons between published protocols and papers challenging. However, there is a multitude of phenotypic tests, such as the expression of liver transcription factors and the asialoglycoprotein receptor (specific for differentiated hepatocytes and analysed by immunocytochemistry, Western blot and cytometry), and functional tests, such as glycogen storage, uptake and excretion of indocyanine green, production of albumin and urea and CYP450 activities, which can be combined to evaluate the efficacy of hiPSC-HEP differentiation. In addition, it should be noted that the control cells, usually cryopreserved hepatocytes, have lower function when compared to that of freshly isolated PHHs, leading to an overestimation of the hiPSC-HEP function [61].

Despite the strategies used and the resulting functions of the hPSC-HEPs, these hepatocytes remain relatively immature, being more comparable to foetal/neonates than to adult hepatocytes [33,62]. This is defined by the limited amount of urea production, a low level of cytochrome p450 activity and/or by their inability to be effectively induced in response to drug induction, when compared to their adult counterparts. Cell polarisation is also an essential feature of functional hepatocytes, which can be examined by immunocytochemistry, whereby specific proteins should only be expressed on apical (e.g. BSEP, MRP-2, P-gp) or basolateral (e.g. CD26, NTCP, OATP) membranes.

However, it is worth noting that "the ideal primary hepatocyte" does not exist *in vitro*. Thus procedures have been defined to cryopreserve only highly viable isolated primary hepatocytes (>80%) and to routinely assess their metabolic function and microbiological safety. This allows for the selection of cells with good metabolic performance and safe hepatocyte batches prior to individual cell transplantations [63]. Moreover, the evaluation of urea synthesis and UDP-glucuronosyltransferase 1A1 activity, allows for the possibility of customizing cell preparations for patients with urea cycle disorders or Crigler-Najjar syndrome type I [64].

Interestingly, it was recently reported that a significantly more efficient and long lasting correction of hyperbilirubinemia was achieved, concomitant with better engraftment, after the transplantation of cryopreserved neonatal hepatocytes into icteric Gunn rats when compared to adult hepatocytes [65]. This suggests that the *in vivo* proliferation and maturation ability of neonate cells are both important properties for cell therapy, and that mature metabolic function is not mandatory for transplantation.

A number of studies from Lola Reid's laboratory and our own laboratory have shown that foetal hepatic progenitors can also mature *in vivo*, and in some models repopulate transplanted livers [66–68]. Furthermore, a recent study showed that injection of endodermal stem/progenitors isolated from human gallbladders into mouse cirrhotic livers resulted in the differentiation and higher repopulation (9.5%) of the cells in comparison to that achieved after the injection of adult hepatocytes (2.4%) [69]. This approach was translated into a clinical trial in which 2 patients with advanced cirrhosis were treated with the above mentioned cells [70].

These properties of the progenitors, which can be easily cryopreserved [45], would avoid the requirement of long term culture. However, to date the only report studying their engraftment showed that hESC/hiPSC-derived progenitors engraft in the liver of transgenic Alb-uPA immunodeficient mice at a very low efficiency (see below) [71]). Receptors, junction proteins and other factors required for engraftment remain to be defined.

Another consideration when using hiPSCs to generate hiPSC-HEPs is the requirement to optimise the differentiation protocol for each cell line being used. Kajiwara *et al.* found that the cells ability to differentiate into hepatocytes was more dependent on the donors, as opposed to the cell type used [72]. The study also showed that their initial stages of the protocol were reproducible and differences in the resulting hepatocytes were a result of varying responsiveness to the maturation cues. This suggests that such differences could be at least partially attributed to genetic variations between the cell lines that may impact the cell response to specific factors, once endoderm specification is achieved by the canonical factors WNT/Activin.

Applications of hiPSC-HEPs

Disease modelling

hiPSCs provide the unique advantage of being generated from any possible cell source including patients with a wide range of disease backgrounds [73]. Two main criteria are vital for the effective use of hiPSCs and their derivatives for

disease modelling. These are i) the adequate exhibition of the required properties of differentiation and maturation that are comparable to the corresponding adult cells, and ii) the ability to accurately recapitulate the main characteristics of the disease itself. An added benefit would be the capacity of the cells to respond to potential therapeutic agents. To date a number of diseases have been successfully modelled, including Alzheimer's disease and amyotrophic lateral sclerosis [74–78]. More specifically regarding liver diseases, models for α1-Antitrypsin (A1AT) deficiency, Wilson's disease (WD), familial hypercholesterolemia (FH), glycogen storage disease type 1a (GSD1a) familial transthyretin amyloidosis (FTA) and Niemann-Pick type C (NPC) disease have been generated. A recent review by Sampaziotis *et al.* [79] provides an in depth discussion of the currently available disease models.

- A1AT deficiency is a monogenic genetic disorder expressed primarily in hepatocytes due to mutations in the SERPINA1 gene that leads to the accumulation of misfolded A1AT in the endoplasmic reticulum (ER), resulting in decreased levels of the protein in extracellular diseases [80]. hiPSC-HEPs were generated from a A1AT patient and the resulting cells showed the accumulation of the misfolded polymers of mutant A1AT in the ER [81]. Moreover a recent study showed that hiPSC can model individual disease phenotypes of patients with A1AT deficiency with or without liver disease in terms of intracellular accumulation and diminished secretion characteristic of the misfolded A1AT protein [44].
- WD is a result of mutations in the ATP7B gene which encodes an ATPase involved in the removal of copper from the body [82]. Generation of hiPSC-HEPs from WD patients was reported [82,83] and the disease phenotype was successfully rescued by overexpressing the wild type form of the ATP7B gene using lentiviral vectors, or through the addition of the drug curcumin [82].
- FH type IIa is an autosomal dominant disease caused by mutations in the LDLR resulting in an abnormal level of circulating cholesterol conjugated to LDL particles [84] leading to severe cardiovascular disease as early as childhood for homozygous patients. The hiPSC-HEPs derived from FH patients (FH-iHEPs) were used to test the effects of lipid lowering agents such as lovastatin and similar results were observed when compared to the effects in FH patients [85]. The diseased phenotype was also reproduced and FH-iHEPs poorly internalized Dil-LDL a fluorescent ligand of the receptor [81]. It is noteworthy to mention that besides LDLR gene, Apolipoprotein B (ApoB) [86] and Proprotein Convertase Subtilin/Kexin 9 (PCSK9) [87], are two other genes responsible for FH pathology. Therefore modelling this disease from patients carrying these types of mutations could enhance our understanding of this pathology and the overall mechanisms involved in cholesterol metabolism regulation. A recent study by Si-Tayeb *et al.* generated hiPSCs with mutations in the PCSK9 gene. The hiPSCs were successfully differentiated into hiPSC-HEPs and assessed for phenotypic attributes related to PCSK9 mutations such as reduced LDL uptake and PCSK9 secretion. The resulting hiPSC-HEPs exhibited such characteristics and treatment with Pravastatin resulted in an increase in LDL uptake and PCSK9 secretion suggesting that this system is useful for studying the effects of PCSK9 mutations [88].
- Generation of hiPSCs from patients with GSD1a and their differentiation into the GSD1a-iHEP resulted in an increase in the accumulation of glycogen and lipid when compared to the wild-type hepatocytes [81].
- With regards to FTA, a disease that affects a number of organs in addition to the liver, there is a requirement of using a number of cell types in order to accurately model the pathology [89]. Leung and colleagues were able to differentiate hiPSCs from FTA patients into hepatocytes, neurons and cardiomyocytes, all of which are involved in the mapping of the disease. Their model demonstrated that hepatocytes did in fact produce the mutant form of the transthyretin (TTR) protein which lead to the induction of oxidative stress followed by cell death in the cardiomyocytes and neurons exposed [89]. Another TTR point mutation leading to FTA phenotype was modelled as well by FTA-iPSCs generation and differentiation into hepatocytes [90].
- NPC disease is another lipid storage disorder with major cerebral and hepatic damages, due to mutation in NPC1, a transmembrane protein located on the late endosomal/lysosomal (LE/L) compartments, leading to sequestration of LDL-derived cholesterol in the cellular LE/L compartments and dysfunctional autophagic flux [91]. Interestingly, Maetzel and colleagues generated isogenic mutant and control cell lines of NPC-iPSCs by point mutation correction using transcription activator-like effector nucleases (TALENs). Generation of isogenic controls from same patient-iPSCs, differing only in one nucleotide, has obvious advantages over control hiPSC lines with completely different genetic and epigenetic profile. This can

highlight the phenotypic differences and identify pathological mechanisms triggered by a given mutation in controlled conditions.

In the majority of hepatic *in vitro* modelled diseases mentioned above, the authors evaluated the effects of a given mutation in patient hiPSC-HEPs, and showed their capacity to reproduce characteristic molecular alterations of these monogenic phenotypes. They are mostly limited to highlighting the low expression, absence or accumulation of the target mutated protein in terminally differentiated hiPSC-HEPs while downstream networks affected by the mutation were neglected. Wilson *et al.* have defined a stage-dependent global transcriptomic signature for A1AT deficiency, showing no difference between patient and control hiPSCs or hESCs at undifferentiated or endodermal stage, and emergence of a pathologic signature with 135 distinguished genes at hepatic stage [92]. These genes, likely representing the downstream effect of accumulated A1AT protein, would be of great interest to study the genetic and epigenetic mechanisms implicated in A1AT deficiency onset.

Stem cell derived hepatocytes have also become invaluable for use in modelling infectious diseases such as hepatitis C virus (HCV) infection [93–95] and malaria in order to establish effective systems to investigate disease progression and assays for drug screening. Various groups [33,96] employed the use of hiPSC-HEPs in investigating HCV infection. Yoshida *et al.* showed expression of receptors needed for HCV infection and HCV replication in hiPSC-HEPs, while HCV entry and viral replication was reduced with anti-CD81 antibody and interferon. Schwartz and colleagues used hPSC-HEP to confirm that the entire HCV life cycle could be sustained within the cells and a resulting inflammatory response was detected. The hPSC-HEP infected with HCV genotype 2a were able to release infectious virions capable of further infecting HuH-7.5 cells in addition to the upregulation of the IL-28B gene expression and TNF-α secretion, both important mediators of the innate immune response. Lastly, Carpentier *et al.* generated hiPSC-HEPs that were successfully infected with HCV and a subsequent immune response could be detected *in vitro*. The resulting hiPSC-HEPs were also susceptible to HCV infection *in vivo* [97,98].

Ng *et al.* demonstrated that hiPSC-HEPs were vulnerable to infection by various plasmodium strains, *P. berghei*, *P. yoelii* and *P. vivax* that cause malaria at the hepatoblast stage [99]. Further maturation of the cells using small molecules allowed the cells to be infected by *P. falciparum*, and treatment with the anti-malarial drug primaquine resulted in reduced parasite infection. The study also highlights the importance of using cell lines with polymorphic variations to assess drug efficacy in various ethnic groups, which is feasible with hiPSC lines.

Regarding *in vitro* modelled liver diseases, all patient-hiPSC-HEPs are differentiated and cultured in static 2D, single cell type conditions. However, intercellular interactions of hepatocytes with non-parenchymal liver cells, as well as cell-matrix interactions are essential to maintain patient-hiPSC-HEPs at *in vivo*-like levels for long term cultures. Improved differentiation protocols providing 3D conformation and co-culture conditions, which will be discussed later, would be key for deeper study of pathology generation mechanisms.

Drug screening and discovery

Drug discovery is a complicated and expensive process; whereby approximately 2 billion USD are required to release a drug onto the market and takes over 10 years [100]. During the recruitment process, 43% of the candidates fail due to lack of effectiveness and 33% due to the formation of negative side effects [101]. Over 40% of the drugs fail at phase III clinical trials [102] because of unforeseen toxicity in late-stage clinical development and post-market release. This is due to our limited capacity to selectively choose the correct candidates prior to use in human clinical trials. Early prediction of ADMET (adsorption, distribution, metabolism, elimination, and toxicity) can facilitate the drug development process.

Expression of key liver enzymes, such as CYP450, decline rapidly after hepatocyte isolation. Cell lines such as HEP-G2, which originated from tumours, also display low levels of the key enzymes (CYP450, conjugating enzymes) as well as low expression of transporters. Furthermore, they do not exhibit polarisation for vectorial drug transport from the plasma to the bile [103]. The current animal models are not accurate due to differences in physiology when compared to humans and primary human hepatocytes (PHH) systems are highly variable and demonstrate limited metabolic function [101]. The ideal tool required for improving the drug screening process is an indefinite source of functional, viable and mature hepatocytes representing donor diversity such as hiPSC-HEPs. However, the fact that current differentiation protocols yield a heterogeneous cell population with varying phenotypes: hiPSC-HEPs that express adult markers (e.g., high levels of albumin expression) while maintaining a simultaneous immature phenotype, raises concerns about the predictive hepatotoxicity. In addition, CYP induction pathways have been shown to be limited in hiPSC-HEPs when compared to PHH, suggesting that nuclear receptors pathways may not be fully functional. Ideally, hiPSC-HEPs should display i) a panel of secreted proteins

such as albumin, apolipoproteins and coagulation factors, ii) full phase I (CYP450) and II (such as UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases, glutathione S-transferases) detoxification functions and iii) adequately express the entire array of basolateral and apical transporters. They should also demonstrate ammonia elimination through urea production.

Despite the fact the drug-metabolizing machinery seems limited, several recent publications illustrate the predictive sensitivity of hiPSC-HEPs to distinguish known hepatotoxins from compounds that do not negatively impact the liver and, most importantly, are capable of generating the relevant toxic metabolites from known hepatotoxic agents (e.g., aflatoxin B1) in sufficient concentrations to reveal their cytotoxic effects [104–106]. In a recent study hiPSC-HEPs were exposed to relevant concentrations of 4 hepatotoxic compounds for 2, 7, and 14 days including amiodarone, aflatoxin B1 and troglitazone. The data showed that the hiPSC-HEPs model was stable enough to enable a 2-week study of drug exposure. The model was also sufficiently specific, shown by evidence of phospholipidosis and steatosis to assess hepatotoxic responses on a mechanistic level. It should be noted that this type of sustained exposure would not be possible with PHHs over the extended culture time [107].

Recent advances have been made to enhance hiPSC-HEPs function; Ware *et al.* modified the micropatterned co-culture technique to include a Matrigel overlay which resulted in improved CYP450 activity and permitted hiPSC-HEPs culture for several weeks necessary for drug toxicity studies. These static co-cultures were treated with a set of 47 drugs and displayed comparable results to those observed with PHH [108]. Another shortcoming of actual *in vitro* biological models used in ADMET prediction of drugs, is the lack of multiple organ metabolism and interactions. Inclusion of 3D co-cultured hepatocytes into a microfluidic device connected with other organs cell types through interconnected channels under physiologically relevant fluid flow conditions, help to understand the effects of crosstalk with other organs-on-a-chip on drug toxicity outcomes [109–111].

Overall, the use of hiPSC-HEPs and other hiPSC-derived cells allows for a diverse set of systems to be developed ranging from high throughput screens for toxicity and metabolic analysis to multi-organ models which more accurately recapitulate drug metabolism in the body through assessing communication between the different organs. Moreover, hiPSC-HEPs can be used to investigate disease progression and isolate possible drug targets for various diseases that affect the liver such as malaria [99], fatty liver disease and hepatitis viral infection described in the above section. Furthermore, the availability of liver disease models will provide unique tools to test the efficacy of drugs used for treatment. This in turn will reduce the variability in the effectiveness of the drug between patients, leading to a more personalised form of medicine. The first successful example of the use of hiPSC-HEPs in drug screening was recently reported using hiPSC-HEPs with A1AT deficiency. A high throughput drug screen was carried out using the Johns Hopkins drug library in order to identify compounds that could effectively reduce mutated A1AT accumulation. The screen resulted in the isolation of 5 possible clinical drugs that exhibited a comparable effect in various patient-derived hepatocyte lines [112].

Lastly, it is worthy to note that the pluripotency of patient-iPSCs is of significant value in drug screening studies; as a monogenic disease can affect multiple target organs. Differentiation of patient-iPSCs to all target cell types will help to overcome cell type specificity of drugs. This was demonstrated by Maetzel *et al.*, where among five autophagy inducers tested, only carbamazepine overcome the impairment in autophagy in both NPC1 iPSC-derived hepatic and neural cells [91].

Cell therapy

Clinical trials involving HT have provided encouraging results [113–116] and therefore suggest that such cell based therapies may represent viable alternatives to OLT. However transplanted cell function appears to often decline after several months, necessitating liver transplantation. The precise aetiology of the loss of cell function is not clear, but poor engraftment and immune-mediated loss appear to be important factors [1]. In 1998, Fox and colleagues were the first to transplant 7.5×10^9 allogeneic hepatocytes into the liver of a patient with Crigler-Najjar syndrome type I, a rare autosomal recessive disorder caused by the absence of the enzyme diphosphate glucuronosyltransferase [UDPGT] resulting in a partial correction of the metabolic disorder for 11 months [117].

Since 1976, various animal models of inborn errors of metabolism have been used, such as the Gunn rats; the animal model for Crigler-Najjar syndrome, to assess the feasibility and efficacy of HT. Animal studies have demonstrated that once injected into the liver, hepatocytes cross the sinusoid endothelial barrier, connect to neighbouring hepatocytes by re-expression of gap junction proteins, survive long term and respond to growth stimuli. However, the majority of infused cells (70–80%) remain entrapped in the portal spaces and sinusoids, and are cleared by the innate immune system, including Kupffer cells and granulocytes. Consequently,

the initial engraftment of hepatocytes post transplantation is low, only corresponding to about 0.5% of the recipient liver mass under normal conditions. In humans, despite successful allogenic cell engraftment, engrafted cells also have a limited capacity to repopulate the liver as they do not proliferate under normal conditions. They are also susceptible to effects of the immune response, in turn reducing graft function over time [118]. These limitations are also a concern with regards to hiPSC-HEPs.

A number of animal models have been developed showing that when transplanted hepatocytes displayed a proliferative advantage over resident hepatocytes they could efficiently repopulate the liver [53]. Two mouse models are often utilised to assess robust engraftment and repopulation of primary human hepatocyte; fumarylacetoacetate hydrolase *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*IL2rγ*$^{-/-}$ and urokinase (alb-uPA) severe combined immunodeficient transgenic mice [119,120]. Among the many strategies that aim to induce a growth advantage/proliferation of transplanted cells, two such techniques should be applicable to humans. One of which is the partial reversible embolization of the portal vein, developed in our own group. This approach and the subsequent transplantation of autologous hepatocyte that expressed a transgene in macaque monkeys resulted in replacement of 6% of recipient liver 3 months after transplantation [121]. Irradiation of the native liver has also been investigated in different animal models and has resulted in increased cell engraftment [116]. A number of these animal models are currently being used to address concerns associated with safety, engraftment potential and functionality, both short and long term of hiPSC-HEPs.

To date, the majority of studies have shown low engraftment percentages of the transplanted pluripotent stem cell derived hepatocytes when compared to primary hepatocytes [122]. However, significant improvement has been achieved over the last 2 years. A recent study carried out by Carpentier *et al.* showed that up to 15% liver repopulation was possible using hiPSC-HEPs in uPA immunodeficient mice [98]. This study demonstrated the highest repopulation percentage to date using hiPSC-HEPs, although in this model 20–50% repopulation was achieved for more than 5 months with PHH [120]. Interestingly *in vitro* staining for CYP2D6 and CYP3A4 was mainly negative and most of the cells remained AFP positive, suggesting that they maintained a foetal phenotype. After transplantation the hiPSC-HEP population underwent further maturation, resulting in downregulation of AFP and expression of CYP450 and could be persistently infected *in vivo* by HCV more than 3 months after injection [98].

Partial correction of metabolic disease model was first achieved with analbuminemic rats after transplantation of hESC-HEPs [53]. A recent study by Chen and colleagues [123] investigated the effects of transplanting hiPSC-HEPs into Gunn rats. In an attempt to drive engrafted hepatocyte proliferation, the host median lobe was irradiated and HGF expressed by injecting adenovirus containing the *HGF* gene 24 h before transplantation. Results showed a reduction of serum bilirubin of up to 60% and hiPSC-HEP liver reconstitution ranging from 2.5–7.5% at long term (4–6 months). It should be noted that primary hepatocytes grafted more efficiently (18%). Overall, this investigation demonstrated the successful use of hiPSC-HEPs to treat metabolic disorders, confirming that the hiPSC-HEPs were able to engraft into the host liver, multiply over time and exhibit normal hepatic function resulting in the alleviation of the disorder. These data also suggest that in metabolic diseases in which hepatocytes are damaged, such as A1AT deficiency [124] or Progressive Familial Intrahepatic Cholestasis [125], cell transplantation should result in more efficient engraftment than in diseases in which damages occur in organs other than liver.

A recent study carried out by Tolosa *et al.* also demonstrated promising results transplanting hESC-HEPs into mice with acetaminophen-induced ALF. The hiPSC-HEPs successfully engrafted and repopulated up to 10% of the host liver. The engrafted cells were able to rescue the disease phenotype, lowering levels of liver transaminases and no tumours were found throughout the period of the investigation [45].

These studies support the use of hiPSC-HEPs for cell therapy. However extensive animal experiments are still required both in small and large animal models, before hiPSCs and their derivatives can be used in human therapy, to address safety issues and to define protocols that promote hepatocyte engraftment.

With regards to normal hiPSC-HEPs, hiPSC-HEP transplantation will be subject to the same challenges of immunological compatibility with the patient as currently experienced with hepatocytes or whole liver transplantation. Generation of normal HLA-typed hiPSC banks (hiPSC Haplobanks) homozygous for HLA-A, -B, and –DR, the most important loci to match, could be a solution to reduce allograft rejection [126]. Interestingly, estimations showed that low number of HLA homozygous donors (between 50 and 150) would provide HLA-compatible tissue for around 50–90% of different populations [127]. Of note, a recently published review discusses the potential use of reprogramming [128] of cells isolated from elder patients. These cells can be rejuvenated through the reprogramming process

Key point

Human induced pluripotent stem cells (hiPSCs), which can both be indefinitely amplified and subsequently differentiated into hepatocytes, have the potential to become a permanent source of quality-controlled hepatocytes exhibiting a wide panel of HLA haplotypes.

itself, as it results in the reversal of changes induced by age such as telomere elongation and DNA methylation patterns and can subsequently be differentiated into hepatocytes.

Developing our ability to use patient-specific iPSCs, gene/cell therapy should also be beneficial as it should avoid issues associated with cell rejection and the need for immunosuppression [3]. Genetic defects can be phenotypically corrected *in vitro* by lentivectors that express the missing or defective genes, or by targeted genome engineering [115]. The therapeutic potential of the resulting cells can then be tested in animal models of inherited human liver diseases, paving the way for "personalised medicine". New strategies of genome editing are now available. A proof of principle study was carried out whereby the mutation responsible for the A1AT disease was corrected in hiPSCs and the derived hiPSC-HEPs were injected into a mouse model of liver injury. The corrected hiPSC-HEPs maintained normal function upon transplantation and no carcinogenic effects were detected [129]. The disease phenotype was rescued using zinc finger nucleases to introduce the normal *SERPINA1* gene in the hiPSC-HEP [129]. The newly expressed protein retained normal structure and function in hiPSC-HEPs derived post correction both *in vitro* and *in vivo*. This study demonstrates the potential of using hiPSCs for personalised cell based treatment. Unfortunately, this only resolves the one part of the disease further investigation is required to full alleviate all the symptoms associated with A1AT. The same disease has been shown to be treated by another targeted gene editing technology of TALENs confirming the feasibility of *ex vivo* gene therapy on patient-iPSCs [112]. Furthermore, this strategy of coupling gene editing using targeted nucleases and autologous cell therapy has gained feasibility with the recent emergence of the clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9 (CRISPR/Cas9) gene editing technology [130] that, compared with the previously used gene editing technologies, is more efficient and allows targeting any sequence in the genome. However this approach is expensive and the technique still remains to be standardized. Furthermore, it is difficult to ensure the same level of quality control (QC) for the hiPSC lines derived "on demand" for a specific patient when compared to haplobanked hiPSC lines which are "on the shelf" with QC already extensively documented.

Liver bioengineering

3D and co-culture techniques

2D cultures do not recapitulate the three-dimensional organization and microenvironment of cells within organs. 2D cultured human primary hepatocytes have been shown to undergo de-differentiation with a loss of epithelial morphology and metabolic activities after few days or even hours [131]. Beside its three-dimensional architecture, the liver is a multicellular organ and hepatocytes are in constant exchange with non-parenchymal liver residing cells including sinusoidal endothelial cells, stellate cells, Kupffer and biliary cells. Many 2D cultures use combinations of primary hepatocytes and other cell types such as; fibroblasts [132,133], liver sinusoidal endothelial cells (LSECs) [134], stellate cells [135], Kupffer cells [136] and mesenchymal stem cells isolated from human umbilical cord or adipose tissue [137] or those isolated from bone marrow [138]. These studies demonstrate longer viability and improved hepatic function when compared to hepatocyte mono-culture. From these observations, it has been concluded that, both the absence of partner cells that are present in the liver and the 2D culture systems likely appear as a limitation for the *in vitro* maturation of hiPSC-HEPs, in addition to the importance of the ability to communicate with their environment, which can be achieved through microfluidisation. As a result, attempts to develop various 3D culture systems including co-culture and/or microfluidisation appear as mandatory [139–142]. 3D cultures on biochips are currently being developed in order to address the need for a relevant cellular model for drug screening. Despite preliminary *in vitro* and *in vivo* assessments, the majority of candidate drugs fail during clinical trials, with a high proportion due to liver toxicity [143,144]. To date, microfluidised liver-on-chips have demonstrated improved ability to maintain the functionality of PHH when compared to 2D cultures allowing for efficient toxicology evaluations [145–147]. Various authors have also described that the culture of the HepaRG cell line (a hepatoblast-like human hepatocellular carcinoma cell line) in microfluidised 3D devices resulted in a more efficient maturation of the cells into hepatocytes, exhibiting higher albumin secretion and P450 cytochrome activity [148,149]. Very recently, Giobbe *et al.* reported the differentiation of human pluripotent stem cells into hepatocytes directly on microfluidic chips [150] further supporting the significant potential of this technology.

The transition from 2D to 3D culture systems not only enhances the function of the hiPSC-HEPs but also promotes their application in BAL devices. The employment of external BAL devices alleviates the pressure placed on the diseased liver which is now allowed to heal with the appropriate treatment [151]. Various BAL systems are currently beginning clinical trials, including the University College London Alginate encapsulated HepaRG [152] and Academic Medical Centre of Amsterdam (AMC)-BAL, also using

Key point

Once differentiated into hepatocytes, hiPSCs from patients with liver diseases can i) model the disease allowing pathophysiological studies and specific drug screening ii) allow personalised cell therapy after genetic correction.

Key point

hiPSC-derived hepatocytes can also be used in external bioartificial livers, in liver-on-chip devices for drug or toxicology studies, as components of bioengineered liver buds or as bio-ink in 3D bioprinters.

the HepaRG cell line [153], which to date is the most effective cell line for application in BAL devices [154]. However, these cells display some chromosomal alterations, which imply functional differences when compared to normal cells. Furthermore, this cell line cannot reflect the normal, inter-individual variability of pharmacokinetics among the patient population. Various studies have confirmed that BAL devices need to provide 10% of total liver function, which requires approximately 10^{10} hepatocytes, assuming that 1 g of liver contains 10^8 hepatocytes [155] and to date, no studies have been published using hiPSC-HEPs in BAL devices.

Interestingly, in 2013, Takebe *et al.* reported the formation of functional and vascularized 3D liver buds obtained from the tri-culture of hiPSC-derived hepatic endoderm (iPSC-HE) with human MSCs and human umbilical vein endothelial cells (HUVECs) [28]. Although the cells were plated in 2D conditions, human iPSC-HEs self-organized into 3D cell clusters called liver buds (iPSC-LB) by an intrinsic organizing capacity that occurred two days post seeding. When iPSC-LBs and conventionally 2D differentiated hiPSCs (iPSC-MH) were transplanted into mice, human vasculatures in iPSC-LB transplants became functional by connecting to the host vessels within 48 h. The formation of functional vasculatures was paralleled with the differentiation of iPSC-LBs, suggesting the importance of three-dimensional and vascularized tissue formation for successful engraftment and maturation at ectopic sites. Although the acquired differentiation level detected in transplanted iPSC-LB was higher than iPSC-MHs, their gene expression profile was still closer to human foetal liver cells. This fact can be explained by the immature state of iPSC-HE used in the experiments. Furthermore, assuming that iPSC-LBs represent hepatic progenitors with the potential to give rise to hepatocytes and cholangiocytes, the presence of cholangiocytes differentiated from iPSC-LBs or biliary ducts was not reported.

Using decellularised whole liver scaffold as a support for cell seeding and whole organ engineering represents another alternative for donor liver transplantation. In decellularised whole liver matrix, microarchitecture and the intact vascular network serves as a route for recellularisation by perfusion of different cell populations. Using this technology, several laboratories have recellularised rodent, rabbit, or pig livers with human or animal cells [156–158] and tried to resolve technical difficulties of transplantation [159]. One of the major challenges to achieve such a functional bioengineered liver is recellularisation and the establishment of functional microvasculature endothelial cell coverage to prevent thrombosis and blood clotting provoked by acellular ECM [160]. In a recent study on a bioengineered porcine liver, endothelial cell attachment was improved by conjugating anti-endothelial cell antibodies to vasculature in order to maximize a uniform coverage of the vessel walls [160]. The re-endothelialised livers demonstrated reduced platelet adhesion upon blood perfusion *in vitro* and were able to withstand the physiological blood flow and was maintained for up to 24 h once transplanted into recipient pigs. The large number of cells required to develop the whole liver is another issue that can be resolved using only one lobe as done by Hussein *et al.* with the decellularised porcine right lobe [161]. Liver bioengineering with patient-iHEPs or hiPSC-derived hepatic progenitors with proliferation capacity could be a solution to provide the required cell numbers needed and also to reduce immunogenicity issues [162].

3D bio-printing is a novel advancement in tissue and organ reconstruction technology, and consists of precise positioning of biological materials, biochemicals and living cells, with spatial control of the placement of functional components to reproduce the complex microarchitecture of the organ [163]. hiPSC-HEPs may be new candidates for bio-printed livers as it has already been demonstrated to be a feasible approach using primary hepatocytes or hepatic cell lines in combination with non-parenchymal cells (endothelial and stellate cells) [164] as well as with matrix encapsulated HepG2 cell line [165,166].

It should be emphasized that more complex, long lasting *in vitro* cell culture models or bioengineered livers containing all liver cell types exposed to repeated drug treatments is required for the detection of *in vivo*-relevant adverse drug effects [143]. Intrahepatic cholangiocytes, as an example, help hepatocytes fulfil their detoxification role by draining and eliminating the metabolites they secrete and the modification of the bile composition. Dianat *et al.* have established a defined protocol for guided differentiation of hESCs and hiPSCs into functional ciliated cholangiocytes expressing CFTR (90%), with bile transport activity and response to different stimuli such as somatostatin, acetylcholine and ATP [167]. Recently, two other teams have established other cholangiocyte differentiation protocols which, applied on hiPSC from patients with Cystic Fibrosis (CF), allowed the modelling of CF biliary failure [168,169]. Tissue vascularization and integration into host circulation is also a vital solution that will enable the transplantation of the bioengineered liver [28]. Furthermore, the requirement of endothelial cells was confirmed in another 3D model by Du *et al.* [139].

Finally the homogeneity of the final product is an important concern. It should be noted that hepatocytes within the liver are known to have

Key point

The functionality of hiPSC-derived hepatocytes has first to be improved using microfluidized, 3D and cocultures; assessment of their safety also has to be achieved, with a special care to their genomic integrity.

varying function and ploidy [170,171]; as such a distinct level of heterogeneity exists. This should be a consideration when using hiPSC-HEPs for *in vitro* applications, whereby the population should be extensively characterized in order to define the level of heterogeneity and thereby accurately interpret the resulting data in the case of drug discovery, toxicology and disease modelling. It may be less of a concern with regards to cell therapy and bioengineered livers as engrafted hepatocytes should express the same functions as their resident neighbour hepatocytes. Another important concern associated with the use of hiPSCs and their derivatives is their genomic integrity and the effects of reprogramming on their genetic stability.

The safety of using hiPSCs and their derivatives in various applications

An overview of genomic abnormalities in hiPSCs

Genomic aberrations in human pluripotent stem cells (hPSCs) have been initially highlighted in hESC lines; with indications of abnormal karyotypes; including recurrent trisomy of chromosomes 12, 17 or X and aneuploidies of subchromosomal regions such as duplications of locus 12p, 17q or 20q11.21 [172]. These recurrent abnormalities may trigger selective advantage during long term culture; for example, the locus 12p contains the gene *NANOG*, a key regulator of the pluripotency network. Contrary to somatic samples, hiPSCs share these equivalent chromosomal and subchromosomal features, and have also been demonstrated to contain regions of uniparental disomy (UPD); occurring when a daughter cell inherits two copies of a chromosome (or part of chromosome) from one parental cell and no copy of that chromosome (or part of chromosome) from the other parental cell [173]. In addition to karyotyping, more resolutive techniques such as CGH-array or SNP genotyping revealed that hPSCs frequently contain regions with copy number variations (CNVs) of various sizes, either deletions or duplications. Moreover, using exome sequencing, Gore *et al.* analysed 22 hiPSC lines and the 9 fibroblast original populations and showed that each hiPSC exome contained an average of 6 single point mutations [174]. From these discoveries, other articles have contributed since to the accumulation of evidences of an important amount of genomic abnormalities in hiPSCs.

In addition to genomic abnormalities, it is well described that reprogramming is also associated with epigenetic changes, such as aleatory reactivation of one X chromosome in long term culture of female hiPSCs [175,176]. Besides, large changes in chromatin organization are observed leading to a global chromatin decondensation triggered by the activation of the genes of the pluripotency network [177]. Thus, some agents inducing chromatin decondensation have been showed to increase reprogramming efficiency such as sodium butyrate [178].

Despite these epigenetic changes, hiPSCs partly maintain an epigenetic memory of the starting cell type, leading to a preferential differentiation potential into the cell types they are derived from [179].

Can mutations in hiPSCs be avoided?

Three hypotheses have been proposed to explain the presence of such mutations in an established hiPSC line. These mutations can i) pre-exist in the initial somatic cell population and be selected during reprogramming either by chance or because, by any mechanism, they confer a selective advantage to the cells; ii) be acquired *de novo* during the reprogramming process; iii) be induced or selected during hiPSC long term culture. The origin of mutations seems to be a combination of these possibilities.

Recent studies using high throughput sequencing showed that at least 50% of the CNVs detected in hiPSC lines (generated from fibroblasts) could also be found, albeit at a very low frequency, in the original fibroblast population [180]. This example highlights the importance of somatic mosaicism, since hiPSC generation requires the cloning and amplification of a single cell genome and this drawback cannot be avoided unless by generating and screening several hiPSC clones from a single individual, possibly varying the somatic cell type of origin since some tissues could be more or less affected by somatic mosaicism [181]. In an attempt to generate the "safest" hiPSCs, non-integrative reprogramming strategies are now available. Despite the fact that the initial studies analysing large cohorts of hiPSC lines did not highlight the link between the reprogramming method and hiPSC genomic integrity [174,182], some evidence has now been reported showing, for example, a positive advantage of mRNA reprogramming strategy over viral approaches [173,183]. However, it seems that the optimal reprogramming method with zero impact on the cell genome is not yet to be established, since reprogramming is a tremendous and challenging process for the cell. In an attempt to overcome this issue, studies have shown that supplementing the reprogramming media with antioxidants resulted in the efficient reduction of genomic aberrations in hiPSCs [184].

Moreover, the number of mutations in a hiPSC line is not stable and long term culture may impact the genomic integrity of the cells. Laurent *et al.* revealed a trend among CNV apparition, describing the recurrent deletions of tumour suppressor genes at early passages and the duplication of oncogenes at late passages [185].

Among the various parameters involved in the long term culture of hiPSCs (i.e. media formulation, use of feeders or synthetic substrates etc.), the choice of the passaging method appeared to be quite crucial. Undeniably, in comparison with mechanical passaging, enzymatic dissociation was shown to be more deleterious for the cell genome [186] and led to the rapid acquisition of supplemental CNVs (even within 5 passages) [187].

The impact of differentiation on the hiPSC genome
hiPSC-based cell therapy relies on the use of differentiated cells generated from hiPSCs, however, little is known about the impact of hiPSC differentiation on the cell genome. A recent study showed that CNVs may appear during the differentiation of hESCs into neuroprogenitors, as rapidly as 5 days post induction of differentiation, this study is the first of its kind [185]. It is important to note that these results were obtained using hESCs at a relatively high passage number 95–96. Using neuroprogenitor differentiation once again as an example, another study demonstrated that differentiated cells contained both CNVs that were already present in the hiPSC line and *de novo* CNVs generated from the differentiation process itself [188]. Use of small molecules such as sodium butyrate, which triggers histone hyperacetylation which in turns increases the solubility of the chromatin, might play a role in genome instability, in particular in cells derived from patients with a DNA repair-deficiency disorder [189].

In our laboratory, we focused on the hiPSC differentiation into hepatic progenitors using our published protocol [167] and showed in three different experiments, that no *de novo* CNVs were triggered during differentiation, which is an encouraging result for hiPSC-based liver therapy [173]. However, the amount of available data is limited and additional studies are required to quantify and verify the potential impact of differentiation on the genomic integrity of the cells. Moreover, the capacity of various differentiation protocols to select specific population of cells (based on their susceptibility and/or survival capacity upon the process) is not understood. Finally, no link is established yet between the complexity/type of the differentiation protocol (number of different steps, substrate used etc.) and the potential to trigger mutations.

What are the barriers for the use of hiPSC in liver cell therapy?
In the context of liver cell therapy, the described mutations found in hiPSCs and their derivatives leads us to address the following question "Do these mutations prevent the use of hiPSC-derived hepatic cells in liver cell therapy?" The answer is a not trivial one. Indeed, the first challenge is to predict the functional impact of the observed mutations. To date, significant research has suggested that thousands of CNVs and SNPs are benign, and mainly contribute to the normal variability of the genome between individuals [190,191]. However, despite the high quality of *in silico* prediction tools, it would be a complex task to certify that any given mutation could be regarded as safe for clinical use. As discussed earlier in this review, the development of hiPSC-based clinical trials may also rely on the generation of banks of hiPSC lines that represent the different haplotypes worldwide, enabling semi-personalised cell therapy and having the advantage to allowing deep studies of the banked hiPSC clones. In this circumstance, the banked hiPSC lines will have to pass an essential step of mutation detection, although the guidelines (the type of genomic analysis methods to be used) and acceptability thresholds have not yet been established. It is important to note that the pioneering hiPSC clinical study in Japan has been stopped. One patient was transplanted in September 2014 with his/her own hiPSC-derived retinal pigment epithelial cells for treatment of macular degeneration, but hiPSCs of the second patient did not pass a genomic validation step. Reportedly, these hiPSCs contained a mutation, potentially in a known oncogene, which is a serious concern (http://www.ipscell.com/2015/07/firstipscstop/). However, it should be noted that the significance of the genomic integrity controls is highly dependent on the application: the use of hiPSC-derived hepatocytes for drug screening, toxicology assays or development of bioengineered devices, will not likely be restricted due the presence of a few mutations in the hiPSC line used or their derivatives, assuming such mutations do not impact the cell function.

All these efforts taken together will allow us to harness the full potential of hiPSCs, hiPSC-HEPs and other cellular derivatives for use in drug screening, bioengineered livers, disease modelling and ultimately cell therapy. As far as cell transplantation is concerned, long term engraftment of hiPSC-HEP in the liver of large animal models will be a prerequisite step prior to clinical trials.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Author's contribution

ZH, CS, ND, AW and ADK prepared the figure and wrote the manuscript.

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