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Functional comparison of adult and pluripotent stem cell-derived hepatocytes

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Abstract

Utilizing hepatocytes as a cell therapy to safely bridge patients from acute liver failure to liver transplantation is limited by its reliance on donor tissue. Pluripotent stem cells can overcome this limitation by providing a pool of self-renewing starter cells, with the capacity to differentiate into hepatocyte-like cells. Here, we apply a published hepatocyte differentiation protocol to GMP grade pluripotent stem cells and compare them with their mature primary hepatocyte counterparts. We show that while ESC- and iPSC-derived iHeps share similar morphological and functional profiles, they have limited functionality when compared with primary hepatocytes. Nevertheless, their ability to produce high levels of alpha-fetoprotein, which share many functional properties with albumin, could be of therapeutic utility.

Introduction

Acute liver failure (ALF) is a rare but potentially fatal illness characterized by an acute onset of severe hepatic injury, resulting in a loss of liver function [1]. Current interventions for ALF involve artificial liver support, which can replace the detoxification functions of the liver but cannot replace the metabolic or synthetic functions [2] [3]. The only intervention for end-stage ALF is liver transplantation. However, with the UK National Health Service reporting an average waiting time of 135 days for a donor's liver to become available, these patients are at risk of acute life-threatening changes in their condition [4]. Therefore, identifying the means to safely bridge patients from ALF to a liver transplant is an important challenge.

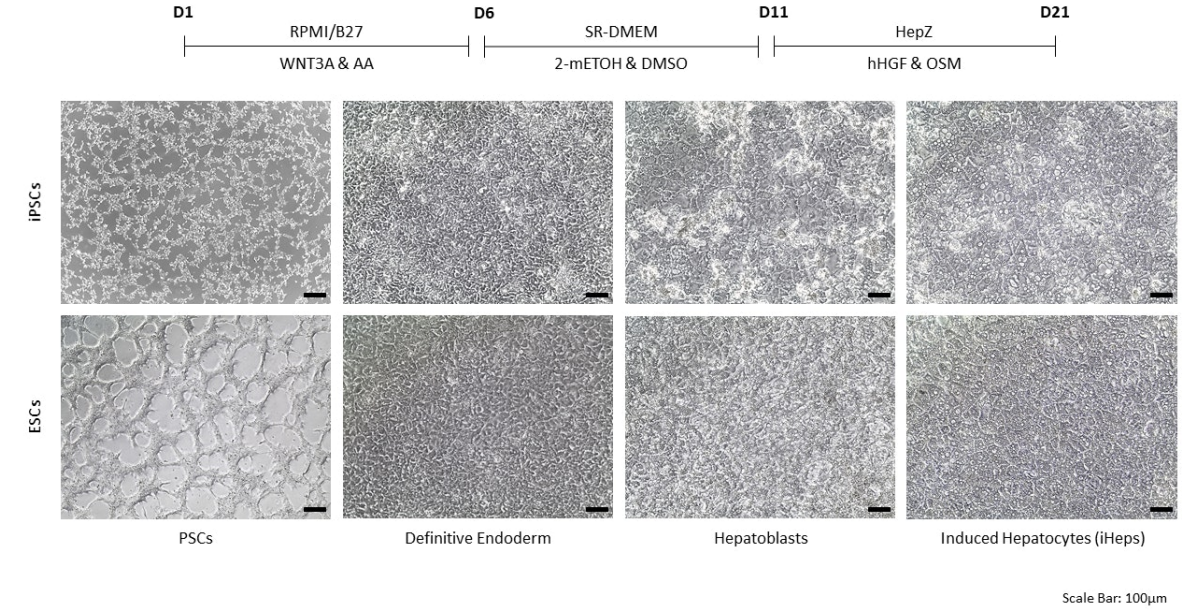
Over recent years, trials using allogenic transplantation of human primary hepatocytes have been demonstrated as an effective bridging therapy for ALF patients, providing the detoxification, metabolic and synthetic functions of the liver [5] [6] [7] [8]. Using allogenic hepatocyte transplantation, however, has two key limitations. The patient still requires immunosuppression and the procedure depends on the availability of donor tissue. Recent progress in GMP-compliant encapsulation methods can bypass the need for immunosuppression, protecting the donor-cells from the recipient's immune response [9] [2]. Nevertheless, the challenge of identifying a sustainable and readily available source of cells that does not rely on the continuous supply of donor tissue remains.

One potential solution to the lack of donor hepatocytes is to use pluripotent stem cells (PSCs) as a source of hepatocytes. Pluripotent stem cells are capable of differentiating into any adult cell type, including hepatocytes, and have self-renewing capacity, providing a pool of replenishing cells [10]. These attributes could, therefore, deliver a sustainable and readily available source of hepatocyte-like cells for encapsulation and therapeutic application, safely bridging patients from ALF to a liver transplant.

Here, we utilize a published differentiation protocol to produce hepatocyte-like cells (iHeps) from human, GMP-grade, pluripotent stem cells [11]. We then compare their function and phenotype with adult primary hepatocytes.

Objective

Several studies have evaluated the ability of PSCs to differentiate into hepatocytes. However, direct comparisons with adult hepatocytes are rarely performed. Our goal was to determine whether the levels of expression of different hepatocyte markers by iHeps was equivalent to adult hepatocytes.



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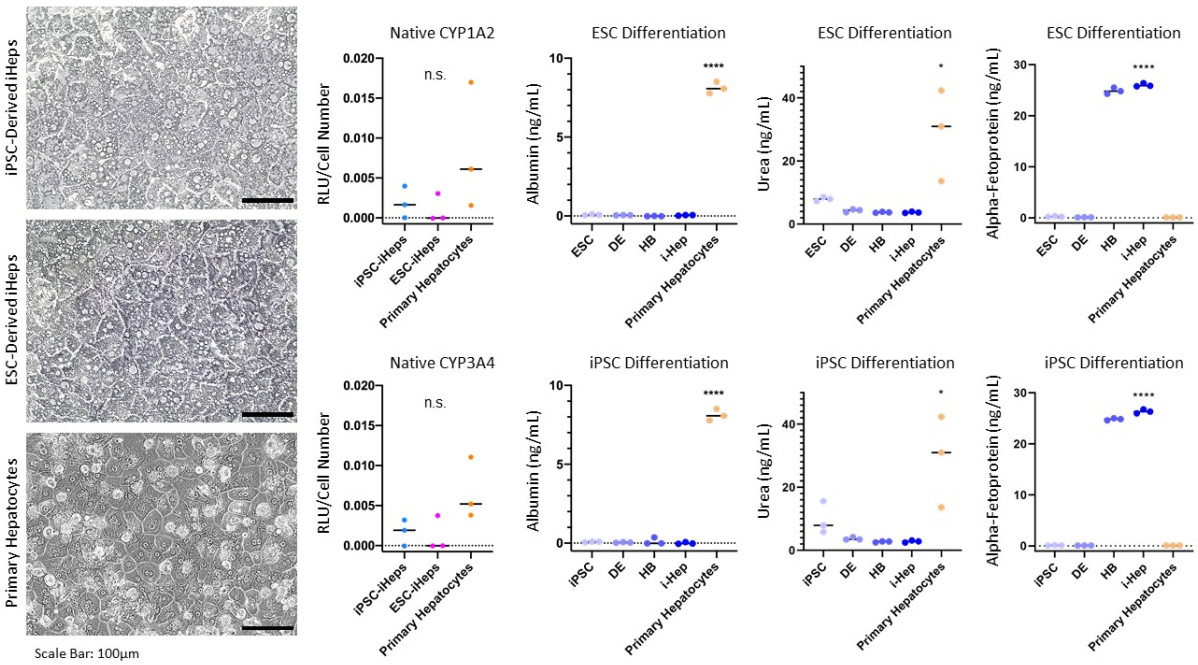

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Figure Legend

Figure 1. Hepatocyte differentiation: morphology and function.

(A) Timeline of the differentiation protocol, showing cell morphology at key stages of the differentiation process.

(B) Morphology of ESC-derived iHeps, iPSC-derived iHeps, and primary hepatocytes; native CYP1A2 and CYP3A4 activity; albumin production; urea production; and alpha-fetoprotein production.

DE: Definitive Endoderm; HB: Hepatoblasts (HB). n = 3 independent experiments. Scale bars: 100 microns.

Results & Discussion

Comparing the morphology of iHeps and primary hepatocytes

We compared the differentiation of two GMP-compliant human pluripotent stem cell lines, one an embryonic stem cell (ESC) line and one an induced pluripotent stem cell (iPSC) line [12] with primary adult human hepatocytes. The morphology of the cells at key stages of hepatocyte differentiation is shown in figure 1A: day 1 (PSCs), day 7 (definitive endoderm), day 11 (hepatoblasts) and day 21 (iHeps). On day 1 the cultures were ~40% confluent, with colony-to-colony contact. By day 7 both PSC lines had reached >90% confluency and exhibited irregular cell shapes, consistent with endodermal morphology. On day 11 both PSC lines had acquired a mixture of irregular and polygonal cell shapes. By day 21 both PSC lines had more defined polygonal cell shapes, with prominent nuclei and the presence of cytoplasmic vesicles, consistent with earlier reports [11] [12].

When comparing the morphology of iHeps and primary hepatocytes, they shared a similar polygonal shape and size. However, the vesicles were less frequent in iHeps than primary hepatocytes (Fig. 1B). We speculate that the vesicles are glycogenic stores, a characteristic of mature hepatocytes.

iHeps retain a hepatoblast phenotype rather than undergoing full maturation

Mature, primary hepatocytes have a variety of differentiated functions, including detoxification, metabolism and protein synthesis [13]. We assessed several functional markers at different stages of iHep differentiation: day 1 (pluripotent stem cells; ESC and iPSC), day 7 (definitive endoderm; DE), day 11 (hepatoblasts; HB) and day 21 (iHeps). We also compared them to mature primary hepatocytes (Fig. 1B).

Cytochrome P450 enzymes are monooxygenases expressed by mature hepatocytes that are involved in a variety of drug metabolism pathways [14] [15] [16]. We measured native cytochrome CYP3A4 and CYP1A2 activity in iHeps and primary hepatocytes, normalized to cell number (Fig. 1B). There was no significant difference in CYP3A4 and CYP1A2 activity between the iHeps and primary hepatocytes.

We next analyzed the levels of albumin, alpha-fetoprotein (AFP) and urea in a 24 h conditioned medium. Albumin functions as a modulator of plasma oncotic-pressure and to transport ligands, including drugs and bilirubin [17]. In contrast to adult hepatocytes, iHeps did not produce a measurable amount of albumin (Fig. 1B). In healthy mature hepatocytes, ammonia is metabolized to urea; urea production was detected in hepatocyte but not iHep cultures (Fig. 1B). Finally we assessed supernatant levels of AFP, which is produced by hepatoblasts but not by mature hepatocytes [18] [19] [20]. At day 11 of the differentiation protocol cells expressed high levels of AFP, indicative of successful hepatoblast differentiation. However, this was retained at day 21, indicating that the maturation had not been achieved (Fig. 1B).

Conclusions

Taken together, the data show that ESC- and iPSC-derived iHeps share similar morphological and functional profiles and have limited functionality when compared with primary hepatocytes. Nevertheless, although AFP is normally only expressed during gestation, as albumin's fetal counterpart, AFP shares many functional properties with albumin [21] [22] [23] [24] [25] and may allow for some clinical application of these iHeps. While primary hepatocytes have been demonstrated to be clinically safe for bridging patients from ALF to a liver transplant, whether these hepatoblast-like cells are sufficient to meet this clinical challenge is unknown. If iHeps can demonstrate sufficient functionality *in vivo*, they may fulfill their bridging task and would have the added

advantage of being more readily available and more consistent in quality than hepatocytes from discarded donor livers.

Limitations

Although the two pluripotent cell lines under study exhibited the same functionality, it is possible that by analyzing a larger panel of lines we would find some with superior hepatocyte differentiation ability. The genetic background of any cell line can influence its behavior in culture.

The native metabolism of ammonia is poor in iHeps, but it was also variable in primary hepatocytes. For a clearer image of the capacity for iHeps to metabolize ammonia, it would be worth challenging the cells with high levels of ammonia and then assessing their metabolic capacity. This is particularly important as hyperammonaemia has been linked to the pathogenesis of hepatic encephalopathy, a potentially fatal complication downstream of ALF.

Alternative Explanations

Conjectures

Our findings suggest that engraftment of iHeps as a bridge treatment in ALF could be beneficial, provided that either hepatoblast function can substitute for the properties of mature hepatocytes or that iHeps undergo maturation into hepatocytes following transplantation in vivo.

Additional Information

Methods

Cell lines and cell culture

Two GMP-compliant human pluripotent stem cell-lines (PSCs) were used: human induced pluripotent stem cell (iPSC) line CGT-RCiB-10 (Cell & Gene Therapy Catapult, London, UK) and human embryonic stem cell (ESC) line KCL037. The CGT-RCiB-10 line was generated from the peripheral blood cells of a female donor. KCL037 was derived from a normal healthy blastocyst. Both cell lines were passaged and maintained on Laminin-521 (BioLamina) coated Corning Costar 6-well plates (Sigma–Aldrich) in mTeSR™1 (STEMCELL Technologies). The cells were passaged every 3–4 days using Gentle Cell Dissociation Reagent (STEMCELL Technologies) in mTeSR1 with the addition of 1:1000 Y-27632 dihydrochloride (R&D Systems).

Hepatocyte differentiation was performed following the protocol [11]. Briefly, PSCs were plated onto Laminin-521 (BioLamina) in Corning Costar 12-well plates (Sigma–Aldrich) in mTeSR™1 (STEMCELL Technologies) at a seeding density of 1×106 per well. On day 1 after colony-to-colony contact was observed, mTeSR™1 was replaced with RMPI medium (Gibco), supplemented with B27 (Life Technologies), Wnt3a (R&D Systems) and Activin-A (Peprotech) and cells were cultured for 5 days. On day 6–10 medium was replaced with DMEM (Gibco), supplemented with Knockout Serum Replacement (Life Technologies), 2-mercaptoethanol (Life Technologies) and dimethyl sulfoxide (Sigma). From day 11–21 medium was changed to HepatoZYME (ThermoFisher Scientific) supplement with human- Hepatocyte Growth Factor (Peprotech) and Recombinant Human Oncostatin M (Peprotech). Cells were assayed on day 21.

Two cryogenically preserved human primary hepatocyte lines were used as positive controls (Lonza 4133 and Lonza 4191). Cells were thawed according to the manufacturer's recommendations (Lonza Suspension and Plateable Cryopreserved Hepato-

cytes Technical Information & Instructions). Both cell lines were plated on collagen-R (SERVA), in Hepatocyte Basal Medium (Lonza) supplemented with HCMTM SingleQuotsTM Kit (Lonza). Both lines were maintained for 7 days, with daily medium changes, before being assayed alongside iHeps.

Microscopy

Brightfield images were obtained using a Leica DMIL LED microscope with a Leica EC3 digital camera. Images were brightened and sharpened using Leica Application Suite LAS EZ and image J.

Hepatocyte functional assays

Cell culture conditioned medium was collected 24 h after a medium change and stored at -20℃ prior to analysis. Alpha-fetoprotein ELISA Kits (Alpha Diagnostic), Albumin ELISA Kits (Alpha Diagnostic) and Quantichrom Urea Assay Kits (BioAssay Systems) were used to assay hepatocyte differentiation. Absorbance for each assay was measured at 450 nm on a Promega GloMax Multi+ Detection System plate reader. Native cytochrome P450 CYP3A4 and CYP1A2 activity were measured in day 21 iHeps and primary hepatocytes using P450-Glo Assays (P450-GloTM CYP3A4 Assay, Promega and P450-GloTM CYP1A2 Assay, Promega). Luminescence was measured for both assays at 450 nm using a Promega GloMax Discover multimode microplate reader.

Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. Comparisons of groups were calculated using ANOVA.

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Ethics Statement

These studies did not involve animals and did not require patient consent.

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