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CELL-BASED DRUG DEVELOPMENT, SCREENING, AND TOXICOLOGY

Developing High-Fidelity Hepatotoxicity Models From Pluripotent Stem Cells

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ABSTRACT

Faithfully recapitulating human physiology "in a dish" from a renewable source remains a holy grail for medicine and pharma. Many procedures have been described that, to a limited extent, exhibit human tissue-specific function in vitro. In particular, incomplete cellular differentiation and/or the loss of cell phenotype postdifferentiation play a major part in this void. We have developed an interdisciplinary approach to address this problem, using skill sets in cell biology, materials chemistry, and pharmacology. Pluripotent stem cells were differentiated to hepatocytes before being replated onto a synthetic surface. Our approach yielded metabolically active hepatocyte populations that displayed stable function for more than 2 weeks in vitro. Although metabolic activity was an important indication of cell utility, the accurate prediction of cellular toxicity in response to specific pharmacological compounds represented our goal. Therefore, detailed analysis of hepatocellular toxicity was performed in response to a custom-built and well-defined compound set and compared with primary human hepatocytes. Importantly, stem cell-derived hepatocytes displayed equivalence to primary human material. Moreover, we demonstrated that our approach was capable of modeling metabolic differences observed in the population. In conclusion, we report that pluripotent stem cell-derived hepatocytes will model toxicity predictably and in a manner comparable to current gold standard assays, representing a major advance in the field. STEM CELLS TRANSLATIONAL MEDICINE *2013;2:000–000*

INTRODUCTION

The process of bringing a drug to market is estimated to take more than 12 years, has high attrition rates, and costs between U.S. $800 million and $2 billion [1, 2]. As a direct result there has been a pronounced reduction in new drug candidates in the pipeline. Furthermore, liver toxicity is the second most common cause of human drug failure, and therefore more predictive assays are urgently required to develop safer and cost-effective medicines. One major bottleneck in safety testing is the routine supply of good-quality primary human hepatocytes from the desired genetic background. The efficient differentiation of pluripotent stem cells (PSCs) offers a way around this, promising a limitless supply of human somatic cells from the required genotype [3]. Using our knowledge in pharmacology, stem cell biology, and materials chemistry, we have developed a highly stable and sensitive model that has serious potential to improve the efficiency of the drug development process.

MATERIALS AND METHODS

Genetic Modification and Characterization of the THLE5B (Tc5) Line

The Tc5 parent hepatocyte cell line was derived by SV40LargeT immortalization of normal (untransformed) human hepatocytes. Clones were then derived from the parent Tc5 line permanently expressing genes for human cytochrome P450s that are responsible for human drug metabolism. The Tc5 cell line was transfected with cytochrome P450 (CYP) 2C9 and CYP2D6 cDNAs, and gene expression was quantified using quantitative polymerase chain reaction (PCR) and compared with human liver. Cytochrome P450 overexpression resulted in increased drug metabolism. CYP2C9 activity was measured using high-performance liquid chromatography for the conversion of diclofenac to 4OH-diclofenac, and CYP2D6 activity was measured by the conversion of dextromethorphan to dextrophen. The human cell line Tc5 and transfected derivatives were incubated with Bristol-Myers Squibb (BMS) 1 and 2 for 48 hours. The selectivity of each compound

was determined in the Tc5 lines. These results were repeated in freshly isolated primary human hepatocytes for BMS 1 and 2. IC_{50} values are quoted in μM.

Human Embryonic Stem Cell and Induced Pluripotent Stem Cell Culture and Differentiation

H9 human embryonic stem cells (hESCs) and 33D6 induced pluripotent stem cells (iPSCs) were reprogrammed, cultured, and differentiated as described in [4, 5]. At day 9 in the differentiation process the cells were replated onto a polyurethane substrate [6] for a further 15–20 days under conditions that support hepatocyte function, changing the medium every 48 hours. CYP1A2, CYP3A, and CYP2C9 activity was assessed using pGlo technology (Promega, Madison, WI, http://www.promega.com) and carried out as per the manufacturer's instructions. Enzyme-linked immunosorbent assays (ELISAs) and immunostaining were performed as described before [4, 7]. Error bars represent the SD (Fig. 1B, 1C).

Primary Human Hepatocyte Thawing and Culture

Cryopreserved primary human hepatocytes (PHHs) (Lonza, Walkersville, MD, http://www.lonza.com) were thawed and replated as described previously [6]. PHH basal drug metabolism was measured using CYP pGlo technology (Promega) as described above. Error bars represent the SD (supplemental online Fig. 1).

Gene Expression Analysis

RNA was extracted using an RNA extraction kit as per the manufacturer's specifications (Life Technologies, Rockville, MD, http://www.lifetech.com), and reverse transcribed to cDNA (Superscript III; Life Technologies). Quantitative PCR was carried out using CYP2C9 primers from Applied Biosystems (Foster City, CA, http://www.appliedbiosystems.com) (Assays-on-Demand) using a 7900HT TaqMan machine. Error bars represent the SD (supplemental online Fig. 2). Statistical significance was determined using Student's *t* test.

Cytochrome P450-Induced Drug Toxicity

To assess the predictive nature of the stem cell-derived models, hepatocytes were incubated with compounds metabolized by specific P450s: BMS 1 and BMS 2. BMS compounds were diluted in dimethyl sulfoxide to obtain a 1,000× stock concentration (50 mM) and were subsequently diluted in media to the denoted final concentration and applied to the cells for 72 hours at 37°C in 5% CO_2. ATP levels within the cells were used as a measure of cell viability at 72 hours post-treatment using CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions. Error bars represent the SD (Figs. 2–4). Statistical significance was determined using Student's *t* test.

Results

Constructing a Stable Model of Hepatocyte Function From iPSCs

Human iPSCs were differentiated to hepatocytes using a highly efficient process [5]. Hepatocellular identity was examined using a panel of human liver genes. Human serum albumin, hepatocyte nuclear factor 4α, α-fetoprotein, E-cadherin, CYP3A, and α-1-antitrypsin expressions were detected by immunostaining in

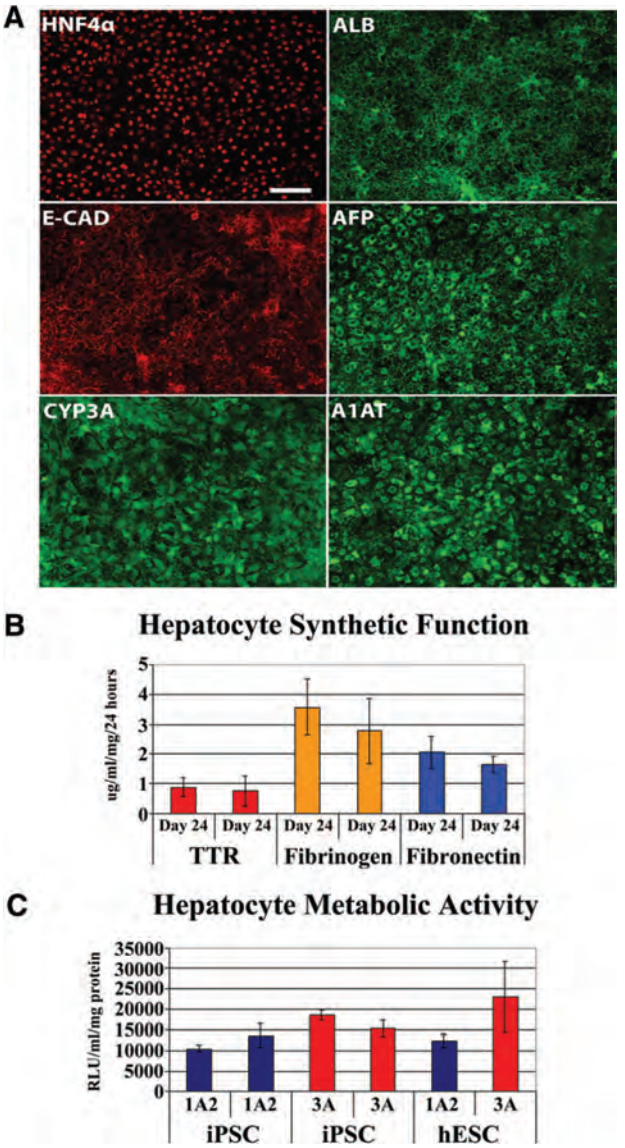
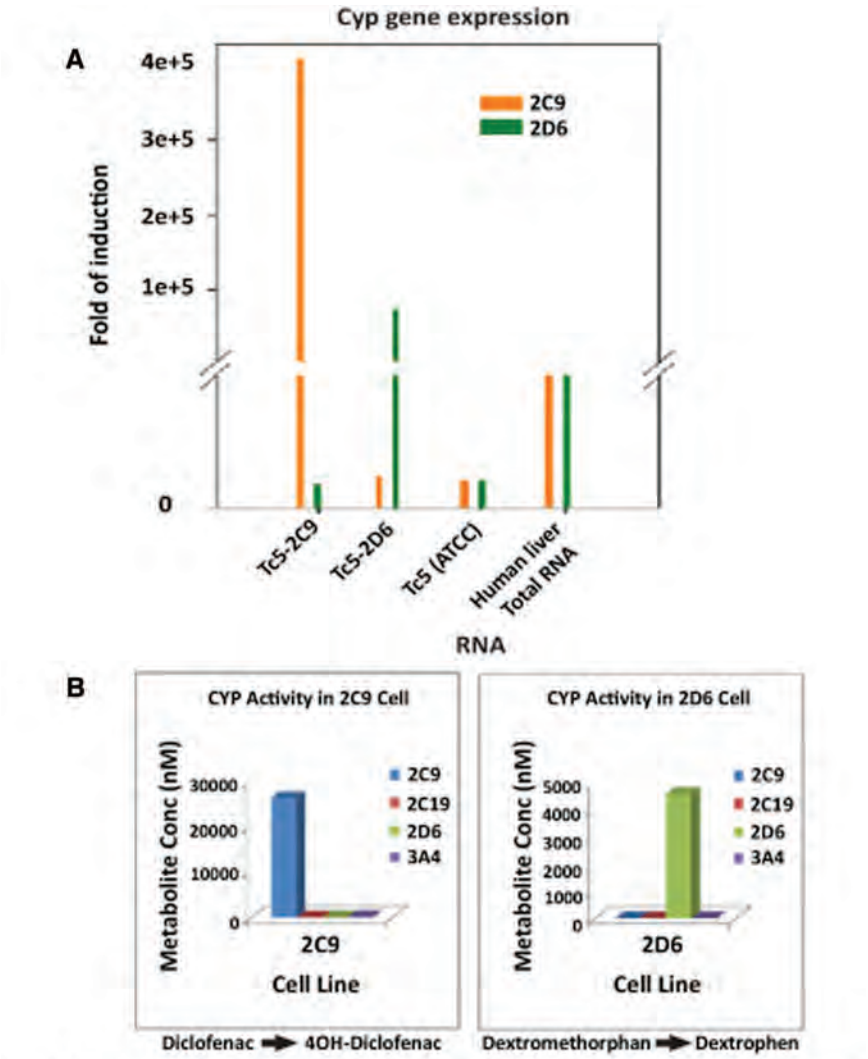


Figure 1. Constructing stable models of human hepatocyte function. **(A):** Human induced pluripotent stem cells differentiated efficiently to hepatocytes using an established procedure and expressed ALB, HNF4a, AFP, E-CAD, CYP3A, and A1AT. **(B):** Human induced pluripotent stem cell-derived hepatocytes displayed hepatocyte function on a polyurethane surface in two separate experiments. TTR, fibrinogen, and fibronectin were detected by enzyme-linked immunosorbent assay as previously described [4]. **(C):** Human iPSC-derived hepatocytes displayed cytochrome P450 drug metabolism (CYP3A and CYP1A2) on a polyurethane surface in two separate experiments in a manner comparable to hESC-derived hepatocytes. $n = 3$ for each experiment; error bars represent the SD. Scale bar = 50 μm. Abbreviations: A1AT, α-1-antitrypsin; AFP, α-fetoprotein; ALB, albumin; CYP3A, cytochrome P450 3A; E-CAD, E-cadherin; hESC, human embryonic stem cell; HNF4α, hepatocyte nuclear factor 4α; iPSC, induced pluripotent stem cell; RLU, relative light units; TTR, transthyretin.

iPSC-derived hepatocytes (Fig. 1A). Previous experiments with iPSC-derived hepatocytes demonstrated narrow windows of peak performance before the onset of cellular dedifferentiation and death. In order to stabilize iPSC-derived hepatocytes, we replated them onto a polymer-coated surface with supportive properties [6]. Hepatocellular protein production and secretion



C

	Compound Selectively Activated/ Detoxified by CYP	Cell lines	Tc5 IC50s				PHH IC50		
		Tc5	Tc5-344	Tc5-2D6	Tc5-2C9	Tc5-2C19	Donor 1	Donor 2	Donor 3
BMS 1	2C9	88.5 ± 18.4	75.8 ± 16.4	95.1 ± 18.9	4.6 ± 1.9	94.3 ± 17.1	>200	>200	>200
BMS 2	2D6	66.9 ± 7.0	78.0 ± 9.6	23.7 ± 3.2	39.3 ± 1.3	68.0 ± 10.4	200.00	19.61	24.22

Figure 2. Identifying a specific and pharmacologically relevant panel of compounds. **(A, B):** Cytochrome P450 gene overexpression was used to deliver metabolically active THLE5B (Tc5) hepatocyte populations. The different clones' metabolic activity was measured using high-performance liquid chromatography. CYP2C9 activity was assessed using the conversion of diclofenac to 4OH-diclofenac, and CYP2D6 activity was measured by conversion of dextromethorphan to dextrophen. **(C):** The human cell line THLE5B and transfected derivatives were used to screen more than 1 million compounds in the search for compounds that were metabolized exclusively by particular cytochrome P450s. BMS 1 and 2 were metabolized exclusively by 2C9 and 2D6, respectively. These experiments were repeated in freshly isolated primary human hepatocytes for BMS 1 and 2. IC_{50} values are quoted in μM. Abbreviations: Conc, concentration; CYP, cytochrome P450.

were assayed using ELISA. Transthyretin, fibronectin, and fibrinogen could be maintained for at least 15 days after cell replating on two separate batches of polymer, indicating significant and reproducible improvements in cell stability (Fig. 1B). In addition to serum protein production, we also examined stable CYP metabolic function. Importantly, iPSC-derived hepatocytes replated on the synthetic substratum not only displayed improved stability but also improved CYP metabolic activity that was more in line with that of human embryonic stem cell-derived hepatocytes (Fig. 1C). These were significant improvements over previous approaches with iPSCs that used biological extracellular matrix for hepatocyte differentiation [5]. iPSC- and hESC-derived hepatocytes were compared with human primary material. iPSC-derived hepatocytes displayed CYP3A and 1A2 activity at levels that were ~34% and ~1% of the levels detected in primary human hepatocytes. This was improved in human embryonic stem cell-derived hepatocytes that exhibited CYP3A function at ~45% of that seen in primary hepatocytes, although there was no increase in CYP1A2 activity (supplemental online Fig. 1A).

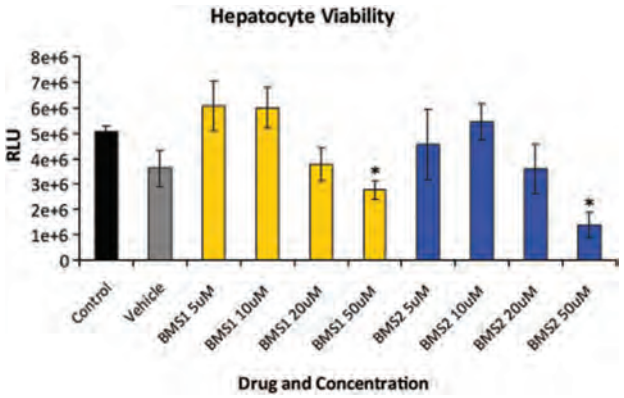


Figure 3. Comparison of stem cell-based model with industry gold standard. To ascertain the sensitivity of stem cell-derived hepatocytes to the BMS compounds, human embryonic stem cell (H9)-derived hepatocytes were incubated with BMS 1 and 2 at different concentrations (0, 5, 10, 20, and 50 μM) for 72 hours. Cell viability was measured at 72 hours after incubation using ATP Cell-Titer Glo. We observed a significant reduction in cell viability for both compounds at a concentration of 50 μM. $n = 3$; error bars represent the SD. Statistical significance was determined using Student's t test; $*$, $p < .05$. Abbreviation: RLU, relative light units.

Constructing a Defined and Pharmacologically Relevant Compound Set

Although stem cell-derived systems displayed hepatocyte function, we wished to establish whether the cells were sensitive to compounds that required metabolic activation. A large compound library (>1 million compounds) was screened for specific CYP metabolic activation and toxic intermediate formation so that functional metabolism could be established. The initial screen was performed in the human liver cell line THLE5B (Tc5) and the overexpressing functional CYP clones, CYP2C9 and CYP2D6 (Fig. 2A, 2B). The Tc5 system flagged two compounds (BMS 1 and 2) that were highly metabolized by CYPs 2C9 and 2D6 to toxic intermediates (Fig. 2C). The toxic nature of BMS 1 and 2 was further confirmed using primary human hepatocytes that had been freshly isolated and represented the current gold standard reference points to which to compare stem cell-derived hepatocyte experiments (Fig. 2C).

Comparison of PSC-Based Models With the Industrial Gold Standard

In order to ascertain the sensitivity of stem cell-derived hepatocyte populations to BMS 1 and 2, cells were incubated with different concentrations of BMS 1 and 2 for 72 hours. After incubation, cell viability was measured using ATP levels as previously described. Stem cell-derived hepatocyte viability was significantly reduced at a concentration of 50 μM for both BMS compounds when compared with the vehicle control (Fig. 3). To further optimize the sensitivity of our system, we extended the differentiation process for a further 4 days (day 20 after replating). The extension in cell maturation resulted in optimized hepatosensitivity to BMS 1 and 2 (Fig. 4A) that overlapped with freshly isolated primary human hepatocytes (Fig. 2C). To examine the reproducibility of our approach from a different genetic background we used iPSC-derived hepatocytes. In comparison with hESC-derived hepatocytes, iPSC-derived hepatocytes exhibited sensitivity to BMS 2. However, sensitivity to BMS 1 was not observed using this cell line. We determined that this was attributable to both reduced CYP2C9 gene expression and reduced function in iPSC-derived hepatocytes (Fig. 4C; supplemental online Fig. 2).

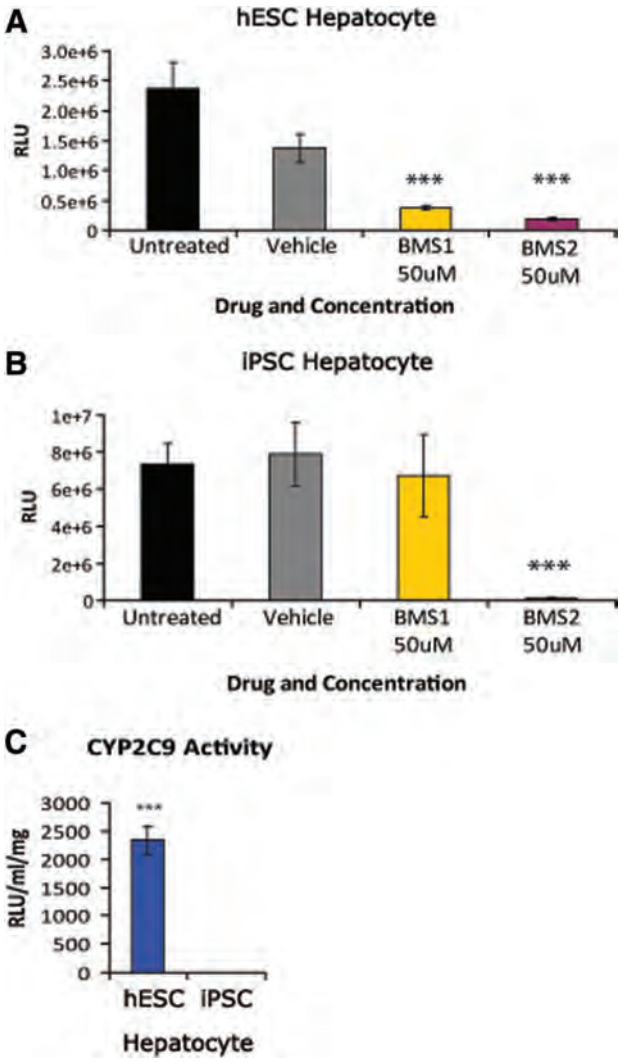


Figure 4. Model optimization, reproducibility, and translation to iPSC-derived hepatocytes. **(A):** Hepatocyte differentiation was extended for a further 4 days to improve sensitivity. Twenty days after replating, human embryonic stem cell (H9)-derived hepatocytes ($n = 3$) were incubated with BMS 1 and 2 at 50 μM for 72 hours. Cell viability was measured using ATP Cell-Titer Glo. Cell viability was significantly reduced in response to BMS 1 and 2. **(B):** Human induced pluripotent stem cell (33D6)-derived hepatocytes ($n = 10$) were incubated with BMS 1 and 2 at 50 μM for 72 hours. Cell viability was measured using ATP Cell-Titer Glo. iPSC hepatocyte viability was significantly reduced in response to BMS 2 and not BMS 1. **(C):** CYP2C9 activity was measured using the pGlo assay. CYP2C9 activity was greatest in hESC-derived hepatocytes (2,341 RLU/ml per milligram of protein). CYP2C9 activity was barely detectable in iPSC hepatocytes at 3.4 RLU/ml per milligram of protein. Error bars represent the SD. Statistical significance was determined using Student's t test; $***$, $p < .01$. Abbreviations: hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; RLU, relative light units.

Discussion

Although media headlines have focused on the development of cell-based therapies from stem cells, the short-term impact will be felt through the delivery of novel in vitro models. With this in

mind, we have taken an approach of generating high-fidelity hepatocyte models from PSC populations that display stable character and provide robust readouts. Our interdisciplinary approach has yielded both stable and improved hepatocyte function on a synthetic surface (Fig. 1). Moreover, cell populations display compound toxicity profiles comparable to those of freshly isolated primary human hepatocytes and model metabolic differences that occur frequently in the population (Figs. 2–4; supplemental online Fig. 2).

Conclusion

These data provide rigorous proof of concept that stem cell-derived hepatocytes have serious potential to improve preclinical assessment of human liver toxicity, especially when the compound is a substrate for hepatic metabolism, and there is potential for toxic reactive metabolite formation [8]. In the future it will be important to screen additional compound libraries using diverse genotypes to gain further insight into the relationship between genotype and drug metabolism [9]. In conclusion, the scalable nature of our model, combined with the interchangeable genetic element, demonstrates clear advantages over the erratic supply of highly variable human hepatocytes from deceased specimens. We believe our approach is important and will likely contribute to improvements in drug safety testing.

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Author Contributions

C.N.M.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; B.L.-V., C.S., F.W., D.S., F.K., and S.P.: collection and/or assembly of data, data analysis and interpretation; J.R.B., H.M.M., and J.A.R.: data analysis and interpretation; M.B.: conception and design; J.P.I., O.F., and D.C.H.: conception and design, manuscript writing.

Disclosure of Potential Conflicts of Interest

D.C.H., H.M.M., J.P.I., and M.B. are founders and shareholders in FibromEd Ltd., UK, and C.S. and O.F. have compensated employment from Bristol-Myers Squibb. F.W. has compensated employment; H.M.M. has uncompensated employment and stock options; M.B. has uncompensated intellectual property rights; and D.C.H. has uncompensated employment, patent holder, and stock options.

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