Richard Siller PhD Thesis

Investigation into *in vitro* hepatocyte models: Method development and applications

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List of Papers Included

1. **Richard Siller**, Sebastian Greenhough, Elena Naumovska, and Gareth J. Sullivan

   Small molecule driven differentiation of human pluripotent stem cells
   (Published Online 30 April 2015, Stem Cell Reports Volume 4, Issue 5, 12 May 2015, Pages 939–952; doi:10.1016/j.stemcr.2015.04.001); Joint First Author with S. Greenhough.

2. **Richard Siller,** Elena Naumovska, Santosh Mathapati, Max Lycke, and Gareth J. Sullivan

   Development of a rapid screen for the endodermal differentiation potential of human pluripotent stem cell lines
   (Submitted, Stem Cells); First Author

3. Wesam Gamal, Philipp Treskes, Kay Samuel, Gareth Sullivan, **Richard Siller**, Vlastimil Srsen, Katie Morgan, Ian Underwood, Stewart Smith, Peter C Hayes, John N Plevris, Pierre-Olivier Bagnaninchi and Leonard J Nelson.

   A human HepaRG-based liver impedance biochip reveals acetaminophen-induced disruption of tight junction and cell-substrate adhesions: An enabling tool for drug hepatotoxicity screening
   (Submitted, Hepatology) Co- Author

Abbreviations

A1AD: Alpha-1-antitrypsin Deficiency

ALF: Acute Liver Failure

APAP: Acetaminophen, Paracetamol

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

BIO: (2´Z,3´E)-6-Bromoindirubin-3´-oxime

BMP: Bone Morphogenetic Protein

CAR: Constitutive Androstane Receptor

cDNA: Complementary DNA

CHIR: CHIR99021, Chemical Name: 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile

CO_2: Carbon Dioxide

CYP450: Cytochrome P450 Enzymes

DE: Definitive Endoderm

DEX: Dexamethasone

DIHEXA: N-hexanoic-Tyr, Ile-(6) aminohexanoic amide

DILI: Drug Induced Liver Injury

DMSO: Dimethyl Sulphoxide

DNA: Deoxyribonucleic Acid

EB: Embryoid Body

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

EGF: Epidermal Growth Factor

ELISA: Enzyme-Linked Immunosorbent Assay

ESC: Embryonic Stem Cells (Human and other species)

FBS: Foetal Bovine Serum

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factor

GF: Growth Factors (in general recombinant human proteins)

GMP: Good Manufacturing Practice

GSC: Goosecoid

GSK3β: Glycogen Synthase Kinase 3 Beta

hESCs: Human Embryonic Stem Cells

HCV: Hepatitis C Virus

HGF: Hepatocyte Growth Factor

hiPSCs: Human Induced Pluripotent Stem Cells

HLCs: Hepatocyte Like Cells

HNF4A: Hepatocyte Nuclear Factor 4, Alpha,

HPLC: High Performance Liquid Chromatography

hPSCs: Human Pluripotent Stem Cells (both embryonic and induced pluripotent)

HTP: High Throughput

IBCA: Impedance-Based Cellular Assay

IDE1/2: Inducer of Definitive Endoderm 1 / 2 (Small Molecules)

iPSCs: Induced Pluripotent Stem Cells (Human or other species)

ITS: Insulin-Transferrin-Selenium

KLF4: Kruppel-like factor 4

KGF: Keratinocyte Growth Factor

KODMEM: Knockout Dubelcco´s Modified Eagle Medium

KOSR: KnockOut™ Serum Replacement

L-15: Leibovitz 15 Media

LSECs: Liver Sinusoidal Endothelial Cells

MEF: Mouse Embryonic Fibroblasts

mEpiSC: Mouse Epiblast Stem Cells

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

miRNA: MicroRNA

MIXL1: Mix Paired-Like Homeobox

MOI: Multiplicity of Infection

MS: Mass Spectrometry

MSC: Mesenchymal Stem Cell

NAPQI: N-acetyl-p-benzoquinone imine

NEAA: Non-Essential Amino Acids

O_2: Oxygen

OSM: Oncostatin M

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PHH: Primary Human Hepatocytes

PI3K: Phosphoinositide 3-Kinase

PPAR: Peroxisome Proliferator Activated Receptor

PS: Primitive Streak

PSC: Pluripotent Stem Cells, from any species, both embryonic and induced

PXR: Pregnane X Receptor

qPCR: Quantitative Polymerase Chain Reaction

RA: Retinoic Acid

RNA: Ribonucleic Acid

RPMI: Roswell Park Memorial Institute Medium

RT-PCR: Reverse Transcription Polymerase Chain Reaction

RT-qPCR: Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction

SI: International System Units

SM: Small Molecule

SNP: Single Nucleotide Polymorphism

TGFβ: Transforming Growth Factor Beta

TJ: Tight Junction

UGTs: UDP-glucuronosyltransferases

U.S.: United States of America

Wnt3A: Recombinant WNT3A Protein

3D: 3 Dimensional

3Rs: Reduction, Replacement, and Refinement (specifically with regards to animal model work and experimental design)

Gene Names

A1AT: Alpha-1-antitrypsin (Serpina)

AFP: Alpha Foetoprotein

ALB: Albumin

APOA2: Apolipoprotein A-II

ASGR1: Asialoglycoprotein Receptor 1

CEBPA: CCAAT/Enhancer Binding Protein, Alpha

CER1: Cerberus

c-MYC: v-myc avian myelocytomatosis viral oncogene homolog

CRIPTO: Teratocarcinoma-derived Growth Factor 1

ERK2: Extracellular Signal-Regulated Kinase 2

FAH: Fumarylacetoacetate hydrolase

FOXA2: Forkhead Box A2

GATA4: GATA Binding Protein 4

HHEX: Hematopoietically Expressed Homeobox

OCT4: Octamer-Binding Transcription Factor 4, also known as POU5F1

POU5F1: POU Domain, Class 5, Transcription Factor 1, also known as OCT4

PROX1: Prospero Homeobox 1

SMAD2/3: Mothers Against Decapentapligic Homolog 2/3

SOX2: SRY (sex determining region Y)-box 2

SOX17: SRY (Sex Determining Region Y)-Box 17

SV40: Simian Vacuolating Virus 40, or Simian Virus 40

T: Brachyury

TBX3: T-Box Protein 3

TDO: Tryptophan 2,3-Dioxygenase

TERT: Telomerase Reverse Transcriptase

TTR: Transthyretin

uPA: Urokinase-type plasminogen activator

WNT: Wingless-Type MMTV Integration Site Family

WNT3A: Wingless-Type MMTV Integration Site Family, Member 3A

ZO-1: Tight Junction Protein ZO-1

SI Units:

Mass:

ng: Nanogram; 1×10^{-9} g

µg: Microgram; 1×10^{-6} g

mg: Milligram; 1×10^{-3} g

g: Gram; 1×10^{0} g

kg: Kilogram; 1×10^{3} g

Volume:

nl: Nanolitre; 1×10^{-9} l

µl: Microlitre; 1×10^{-6} l

ml: Millilitre; 1×10^{-3} l

l: Litre; 1×10^{0} l

Distance:

nm: Nanometre; 1×10^{-9} m

µm: Micrometre; 1×10^{-6} m

m: Metre; 1×10^{0} m

Concentration:

nM: Nanomolar; 1×10^{-9} M

µM: Micromolar; 1×10^{-6} M

mM: Millimolar; 1×10^{-3} M

M: Molar; 1×10^{0} M

Temperature:

^{o}C: Degrees Celcius

Time:

s=Second

Other:

g: Standard Gravity Value; 9.80665 m/s^2

Introduction

The liver is an essential organ, which performs multiple functions including homeostasis, secretion of serum proteins such as albumin (ALB), fibrinogen, fibronectin, alpha-1-antitrypsin (A1AT), as well as the metabolism of xenobiotics. There are a number of cell types that make up the liver: hepatocytes, cholangiocytes, hepatic stellate cells, liver sinusoidal epithelial cells, as well as Kupffer macrophages (see Fig. 1) [1]. There are numerous diseases associated with the liver, caused either by genetic defects or by xenobiotic insults to the liver. Examples of genetic based liver diseases include cystic fibrosis, familial amyloidotic polyneuropathy, alpha-1-antitrypsin deficiency (A1AD), Wilson's disease, familial hypercholesterolemia, glycogen storage disease type 1a, and Alpers-Huttenlocher Syndrome among others (see review of inherited liver diseases [2]). Liver injury as a result of external insults are a major burden on the health care sector, exemplified by drug induced liver injury (DILI), alcoholic liver injury, as well as infectious agents such as malaria and Hepatitis C virus (HCV). The liver is an interesting organ due to its high capacity of regeneration after toxic insult. This regenerative capacity has been known since antiquity, and was immortalized in the myth of Prometheus. In this myth, Prometheus angered Zeus and was punished by being

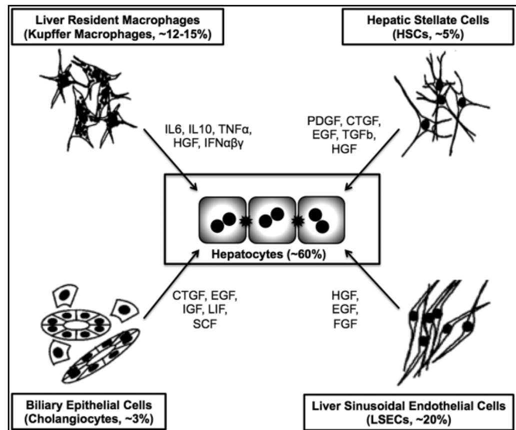

Figure 1 From Khetani et al (2015) [1] showing the complex cell types that form the liver and their interactions.

chained to a rock and having his liver eaten daily by an eagle, only to have it regenerate every night [3].

This thesis will present several critical aspects of liver biology, stem cell differentiation, and the development and use of novel *in vitro* toxicology models. In short, (i) we have developed a novel methodology for the differentiation of human pluripotent stem cells (hPSCs) into functional hepatocyte like cells (HLCs); (ii) designed a screen to rapidly assess multiple hPSC lines for their capacity to differentiate to endoderm, the starting point for HLCs; and (iii) using the well-established cell line HepaRG, have developed a novel toxicity-testing platform for assessing drug metabolism in real time and in a non-invasive manner. An exciting outcome of this work was the identification of a previously unknown aspect of acetaminophen (APAP) induced hepatotoxicity.

There are several major bottlenecks in the current paradigm of pharmaceutical development which cause drug attrition, significantly increasing the time and cost to bring a new medicine to the market. Before a drug reaches clinical trials it must be assessed for efficacy and toxicity in numerous animal models. Unfortunately animal models do not always faithfully recapitulate human physiology, as a consequence a number of key parameters cannot be reliably determined, for example, adverse drug reaction and indeed the efficacy / potency of new drugs. Many of these aspects cannot be fully verified until clinical trials are commenced. One of the primary causes of novel pharmaceutical products failure is the development of severe toxicity in patients, so called ‘adverse reaction,’ primarily affecting the heart and liver. The ability to predict these unwanted side effects would be hugely beneficial, as a consequence, a number of experimental models have been developed and are currently

employed to screen large number of potential drugs. However, many of these experimental models are animal based, with animal studies being a pre-requisite before potential pharmaceuticals reaching Phase I trials in human subjects. It should be noted that animal models have provided a powerful platform for testing the physiological effects of a drug in the context of a whole organism's *in vivo* situation, but in recent years there have been increasing pressure to reduce the amount of animals used for drug testing based on the guidelines of the 3Rs: reduction, replacement and refinement [4]. Unfortunately, *in vitro* cell based models are not predictive of secondary adverse reactions caused by drug metabolism such as rashes and whole organ system failure.

Drug-induced hepatotoxicity accounts for approximately 50% of acute liver failure (ALF); while the majority of these are caused by misuse of drugs, nearly 16% of cases are caused by pharmaceutical drugs and are idiosyncratic in nature, representing a significant burden to both industrial pharmaceutical development as well as to clinical practices [3, 4, 5]. Although many of the hepatotoxic effects of drugs are conserved across many laboratory animal species, including rodent models, idiosyncratic hepatotoxicity is often difficult to predict in animal models which do not fully recapitulate the human physiology [5, 6]. Testing of novel pharmaceuticals in animal models has a long history, but there are several major drawbacks to these studies. Importantly, a 2007 publication by the Committee on Toxicity Testing and Assessment of Environmental Agents, part of the U.S. National Research Council, entitled *Toxicity Testing in the 21st Century: A Vision and a Strategy,* called for a dramatic shift away from qualitative *in vivo* animal studies, towards more quantitative *in vitro* approaches combining automated robotic technology with established human primary cells, as well as cell lines [4]. There are

several critical reasons for this paradigm shift away from animal models. While animal models can provide much information about how a novel compound is metabolised and how it affects / interacts with multiple organ systems in a living system, they often do not recapitulate the full complexity and physiology of a human. The reasons for this lack of concordance are complex. In general, animal models of hepatotoxicity use higher concentrations of the drug (mg/kg), than would normally be used in the clinical setting for humans [4]. A further complication is while inbred strains of rodents are extremely useful for genetic studies, they often do not recapitulate the genetic diversity and heterogeneity of human populations [8]. Perhaps an obvious, but necessary statement is that while rodents and humans share many conserved physiological and genetic features, the rodent system is often incapable of fully recapitulating the human physiology [9]. This is especially relevant for cases of idiosyncratic DILI, being one of the major causes of drug attrition during both development and clinical trials. To complicate the situation further, animal studies generally only focus on a single pharmaceutical compound at a time, whereas in the clinical setting a patient will often be exposed to numerous drugs concomitantly [4]. The number of combinations and critically the number of control experiments required would significantly increase the length of studies. This in turn would drive costs of animal studies higher, thus preventing this from being implemented on a meaningful level. Considering the major issues associated with animal models, it is critical to develop carefully planned *in vitro* studies, which can provide accurate screening of lead compounds. Furthermore, these assays must be verified *in vivo* to confirm their efficacy before animal studies can be replaced or at least reduced in compliance with the 3Rs: reduction, replacement and refinement.

Drug Metabolism in the Liver

Drugs are primarily processed and metabolised in the liver in order to form easily cleared hydrophilic products. Generally this biotransformation results in the production of molecules which are more polar than their parental drug molecule [10]. While the primary benefit of biotransformation and metabolism of a drug is that the resulting metabolites are more easily eliminated from the body, the metabolites often have significantly different biological functions and toxicological properties than the original drug. There are two primary classifications of enzymatic reactions which occur in the liver: Phase I, oxidative processing of drugs, mediated by the cytochrome P450 (CYP450) enzymes; and Phase II, the conjugative metabolism of drugs, mediated through UDP-glucuronosyltransferases (UGTs) [10]. Both the Phase I and Phase II enzymes are primarily localized in the endoplasmic reticulum. Phase I, CYP450 mediated metabolism will be dealt with in depth below, but in general catalyze the oxidation, reduction and hydrolysis of drug molecules. Phase II enzymes mediate the addition of various biological groups to the metabolites from Phase I processing of drug molecules through various enzymes such as UDP-glucuronosyltransferases, sulphotransferases, and glutathione S-transferases. The end result of both Phase I and Phase II reactions is the addition of polar groups to the drug molecule which facilitates its secretion and ultimately its elimination through the biliary system and eventual expulsion through urine.

Phase I Metabolism: The Cytochrome P450 Enzymes

The CYP450 family of proteins are the primary enzymes responsible for Phase I metabolism of pharmaceutical drugs. The CYP450 enzymes metabolise drugs through oxidative metabolism, whereby drugs are converted through the binding of two oxygen atoms, resulting in a more polar metabolite and water as a byproduct [11]. Often, the secondary metabolites are responsible for the deleterious effects of drugs, as is the case for the metabolism of acetaminophen [8]. Most CYP450 based metabolism occurs in the liver, which has the highest concentration of CYP450 protein of all the tissues of the body. The intestinal epithelia also performs CYP450 mediated drug metabolism and clearance, which can substantially modulate the level of a drug absorbed into the blood stream [12]. It is important to highlight that there is significant genetic variation amongst individuals, which directly affect CYP450 activity and contribute to idiosyncratic DILI, differential drug responses and can contribute to the development of cancer [7, 13, 14, 15].

Animal Models

Currently there are numerous animal models for hepatotoxicity, with the primary focus being on mouse, rat, canine, and monkeys. Smaller animals, such as rodent models are useful because they have a short life span with a rapid progression to reproductive age, which facilitates the breeding of a large number of animals in order to perform many studies. Additionally, both rats and mice are available in highly in bred strains. As a consequence, the genetic diversity which can affect the experimental outcome can be tightly controlled. However, larger animal models such as canine (primarily beagle) and monkey, are more

physiologically relevant to humans and generally demonstrate similar pharmacokinetic properties to man [16, 17]. In addition, due to their longer lifespan, large animals can provide the ideal platform for long term, longitudinal studies for assessing drug safety and efficacy [4].

Although the necessity and relevance of animal testing is generally supported, there are critical differences in the metabolic activities between different species, which makes it difficult to meaningfully extrapolate results obtained in animal models to the human situation upon administration of drug. The most relevant differences in terms of hepatotoxicity are related to specific differences with respect to CYP450 activity, induction and inhibition. For a complete review of species specific differences in CYP450 activity, see the review by Martignoni and colleagues [9].

Two of the most relevant CYP450 enzymes involved in drug metabolism and responsible for adverse drug reactions, are CYP1A2 and CYP3A4. CYP1A2 accounts for approximately 13% of the total human liver CYP450 content, and its activity is responsible for up to 4% of all observed drug metabolism [7, 12, 18, 19]. Due to its importance in human drug clearance, there is significant interest in testing CYP1A2 activity in animal models prior to clinical trials. However, there are critical differences in CYP1A2 activity with respect to its inducibility across species. Animal models such as dog and monkey present with much lower endogenous level of CYP1A2 as compared to human [20, 21]. Additionally, the CYP1A2 response to challenge by drugs is different between rodent and human. For example, in human the drug omeprazole (which is commonly prescribed to treat gastroesophageal reflux disease) is a potent CYP1A2 inducer, while in rodents omeprazole challenge does not significantly induce the expression or activity of CYP1A2 [22–25].

Probably the most important CYP450 subfamily is the CYP3A enzyme family. CYP3A enzymes are involved in the metabolism and clearance of over 50% of pharmaceutical drugs currently available [26]. There is substantial interspecies variation in CYP3A subfamily activity in response to drugs. A well-documented example is in the processing of the small molecule rifampicin, which is used primarily in the treatment of bacterial infections such as tuberculosis. In both human and dog, rifampicin is a potent inducer of CYP3A4, mediated through the Pregnane X Receptor (PXR), whereas in rodent models such as the rat and mouse, rifampicin does not induce CYP3A4 [20, 24, 27] This is likely due to species specific differences in the PXR ligand binding [8].

The observed interspecies variation with respect to drug metabolism exhibited amongst different animal models, highlights the importance of experimental design with respect to choice of the appropriate animal model when studying drug toxicity. In light of these important differences, great effort has been expended to create novel *in vivo* models that can recapitulate human physiology more accurately. This has lead to so-called humanised strategies for achieving improved correlation between animal and human biology. For example, there have been knock in approaches wherein the mouse CYP enzymes have been replaced with the human isoforms. Additionally, humanised mouse models have been created through the ablation of mouse liver through genetic approaches such as the urokinase-type plasminogen activator (uPA) model where uPA is under the control of the mouse albumin enhancer / promotor or the Fumarylacetoacetate hydrolase (FAH) knockout mice lines and subsequent induction of liver injury [182]. By ablating the mouse hepatocytes, followed by repopulation of

the liver with human hepatocytes, a closer approximation to human hepatic function can be achieved and studied.

A New Paradigm in Toxicity Testing

To help alleviate the current problems associated with *in vivo* testing of drugs, and to gain a more comprehensive, as well as predictive assessment of potential risk to patients, the U.S. National Research Council has outlined 4 major criteria which should be applied to develop faithful models of toxicity (for overview see Soldatow et al (2013) [28]). Briefly, the 4 criteria are: 1) In order to provide improved models to assess the complex interactions between new pharmaceuticals and commonly prescribed drugs (commonly prescribed concomitantly), toxicity assays must be capable of assessing multiple compounds together. Additionally, the system should take into account both duration of treatments, as well as different 'life-stages' at which the compounds will be used. 2) In order to provide more cost effective assays, it is important to decrease the length of time that possible risks will be identified through use of the novel toxicity assays. 3) In accordance with ethical guidelines for studies that use animals, the number of animals and their potential or realised suffering must be reduced as much as possible. 4) Finally, every effort should be taken to extrapolate the mechanistic cause of toxicity in relationship to the relevant dose response curves. This point is of critical importance and is the most challenging.

***In Vitro* Models of Hepatocytes**

In order to address the concerns raised with the use of animal models of hepatotoxicity, there is increasing effort to develop *in vitro* cell line based hepatic models which faithfully

recapitulate the metabolic capabilities and toxicological responses of the *in vivo* organ. There are a number of benefits to be gained using *in vitro* approaches, with cost reduction being a major driver. Additionally, *in vitro* modeling allows the development of high throughput hepatotoxicity approaches facilitating the screening of large numbers of potential pharmaceutically active small molecules in a shorter time period and reduced cost. While *in vitro* models provide many potential benefits, it needs to be highlighted there are associated limitations with many of the *in vitro* models, mainly due to reduced function compared to an organ. Below, a number of *in vitro* hepatotoxicity models will be described and assessed in terms of their utility and limitations.

Primary Hepatocytes: Attributes, benefits, limitations

Primary human adult hepatocytes (PHHs) are considered to be the gold standard for *in vitro* hepatotoxicity screening. When they are freshly isolated from liver biopsies, they have the morphological and functional attributes of their *in vivo* counterpart. However, there are a number of significant limitations with respect to their routine use. Normally, PHHs are isolated from tissue resections from patients with liver disease or cancer and in most cases the highest quality organs are reserved exclusively for transplantation rather than *in vitro* testing due to ethical reasons. Additionally, PHHs tend to originate from a single donor, thus genetic variation can severely limit translation of any findings to the population as a whole. Furthermore, the lifespan of freshly isolated PHHs *in vitro* varies dramatically, from several hours to several days, which greatly restricts their usage. Longitudinal studies and repeat dosage studies as a result cannot realistically be performed, further limiting their utility.

The isolation procedure can play a significant role in the quality of PHH cultures. The majority of protocols use perfusion of the liver with collagenase to disrupt the cells and isolate hepatocyte suspensions. This disruption inherently destroys cell-cell and cell-extracellular matrix (ECM) structures, which result in destruction or damage to cell membranes, cellular junctions as well as critical cell membrane transporters [28–30]. The loss of these critical cell-cell and ECM interactions during PHH isolation is believed to cause the observed rapid de-differentiation, reflected in significant loss of expression / function of many drug transporters and CYP450s. Although these interactions can be partially re-established and maintained for periods of time *in vitro*, through the use of sandwich cultures and three dimensional (3D) organotypic models, the observed function is significantly lower than *in vivo*. A further complicating factor with the use of PHHs for *in vitro* drug toxicity testing is a lack of a reliable source of cells, which is further compounded by inter-donor genetic variability.

Immortalised Hepatocyte Cell Lines

Due to the above problems associated with PHH culture and maintenance, several immortalised human hepatic cell lines have been derived and characterised. Several approaches have been used to immortalise PHHs, most prominently the use of either Simian vacuolating virus 40 (SV40) Large T or telomerase reverse transcriptase (TERT). A number of lines have been developed using these approaches, including the cell line Fa2N-4 [31]. However, many of these cell lines have limited hepatocyte-like function and are prone to genomic instability. Fa2N-4 is a useful line, as it possesses various drug metabolising properties, however it use as an hepatic surrogate for toxicity screening is severely limited due to extremely low levels of expression of the nuclear constitutive androstane receptor (CAR) as well

as several other transporters compared with freshly isolated PHHs [33]. CAR is a major pathway for drug metabolism in hepatocytes, and a lack of activity severely limits its utility. In addition, during the process of cell line maintenance, varying degrees of CYP450 activity are often observed; thus the activity of this line must be verified regularly before any meaningful data can be assessed [28].

In order to overcome the shortfalls of artificially immortalised hepatocyte cell lines, there have been efforts to establish and characterise various liver cancer cell lines, derived from patient biopsies. This approach has progressed with varying degrees of success. A number of cell lines have been generated from tumours, such as Hep3B, Huh7, HBG, and HepG2 lines[33]. While these lines have varying degrees of hepatocyte like activity *in vitro*, their hepatic function decreases over time, and do not provide a stable hepatocyte like phenotype [34]. Additionally, subclones of these hepatoma cell lines have been derived which present with higher hepatic function [35, 36, 37]. While many of these lines have inducible CYP450 expression and function, again these present with karyotypic instability when cultured at high confluence as required for hepatic maturation, therefore they do not provide a stable alternative for *in vitro* models of hepatotoxicity [33].

HepaRG—An Improved Cell Line

Probably the most extensively used and best characterised cell line for *in vitro* hepatotoxicity modeling is HepaRG. The HepaRG cell line was originally isolated from a female patient suffering from a hepatoma [10]. These cells retain many critical hepatic functions including liver-specific glycolysis and CYP450 enzyme expression, which is inducible on

xenobiotic insult, at higher levels than other immortalised HLC lines. In contrast to lines such as Fa2N-4, HepG2, Hep3B and Huh7 described above, HepaRG cells have active CAR, PXR and peroxisome proliferator-activated receptor (PPAR) comparable with PHHs, making them the most robust and widely used tool in hepatotoxicity screening currently available [11–14]. In addition, HepaRG express the major CYP450 enzymes at robust levels and demonstrate inducibility when challenged with known pharmaceuticals [36, 39, 40].

HepaRG is an ideal cell line for *in vitro* toxicity modeling because they are relatively karyotypically stable [10]. HepaRG cells are a bipotential cell line capable of trans-differentiation to both HLCs and biliary cells (cholangiocytes) depending both on media composition, signaling, and the confluency at which they are cultivated [15]. This increases their utility beyond metabolic drug assays into the realm of choleostatic and whole liver toxicity assessments. An important caveat is that HepaRG is a cell line derived from a single donor, representing a single genetic background, and as a consequence the lack of genetic diversity significantly limits the translations of any findings to other genetic backgrounds.

General Discussion on Human Pluripotent Stem Cells

Due to the lack of secured supplies of quality PHHs, combined with the lack of genetic diversity in both PHHs / immortalised lines for *in vitro* modeling of adverse toxicity, the field has gained momentum to look at alternative sources of material. Such an alternative is the use of human pluripotent stem cells (hPSCs), which have gained the interest of both the pharmaceutical industry and basic science researchers alike. Below, the basic biology of hPSCs, both embryonic (hESCs) and induced pluripotent stem cells (hiPSCs), their application and differentiation to HLCs is reviewed.

Human Embryonic Stem Cells

hESCs are pluripotent stem cells derived from the inner cell mass of discarded blastocysts fertilized *in vitro* [41]. hESCs have two unique attributes, (i) the ability to proliferate indefinitely *in vitro* and (ii) pluripotency, the ability to differentiate into cells representative of all three embryonic germ layers: mesoderm (such as cardiomyocytes [42–44]), ectoderm (such as neurons [45–52]) and endoderm (such as hepatocytes [53–62], pancreatic beta cells [63], lung epithelial cells [64] and intestinal cells [65]). hESCs morphologically are characterised by a high nuclear:cytoplasmic ratio, growing as flat colonies *in vitro*. Despite their potential for both regenerative medicine and basic studies of developmental biology *in vitro*, hESCs are limited in their use due to ethical considerations associated with the destruction of human blastocysts during their derivation.

Human Induced Pluripotent Stem Cells

Many efforts have been employed to overcome the limitations posed by the use of hESCs. In 2006/2007, Shinya Yamanaka and colleagues demonstrated, initially in mouse, the remarkable ability of just four transcription factors to convert (reprogramme) terminally differentiated cells (fibroblasts from a skin biopsy), into so-called induced pluripotent stem cells (iPSCs) [66]. These iPSCs were generated initially by screening a large number of transcription factors known to be highly upregulated in embryonic stem cells. When the complete library of transcription factors was integrated into the genome of the somatic cells via retroviral mediated gene transfer, a small number of pluripotent stem cell like colonies appeared and could be subsequently picked and cultivated. Further work demonstrated that only 4 of the initial factors were critical for the generation of iPSCs: namely POU Domain, Class 5, Transcription Factor 1 *(POU5F1,* also known *as OCT4),* SRY (sex determining region Y)-box 2 *(SOX2),* Kruppel-like Factor 4 *(KLF4),* and v-myc avian myelocytomatosis viral oncogene homolog *(c-MYC).* The resulting iPSCs shared the features of their ESC counterparts, including tri-lineage differentiation potential *in vivo* through teratoma formation and *in vitro* via embryoid body (EB) formation. Additionally, these cells were capable of tetraploid complementation (the gold standard for pluripotency), giving rise to pups derived completely from the injected iPSCs, proving their pluripotency further [67]. This was a major milestone, which culminated with award of the Nobel Prize (2012) in Physiology or Medicine to Yamanaka and John Gurdon.

Subsequent work by a number of groups demonstrated that the process of cellular reprogramming could also be applied to human somatic cells [68–71]. This major milestone

opened the door for research into regenerative medicine, building on previous work with hESCs. A major advantage of working with hiPSCs is that the ethical issues associated with the destruction of human blastocysts are circumvented. Importantly, hiPSCs can be derived from a wide range of genetic backgrounds, enabling the creation of biobanks for personalised medicine. Furthermore, iPSCs can be derived from diseased patients and thus can be utilized to model human disease *in vitro* [68, 72] (Fig. 2).

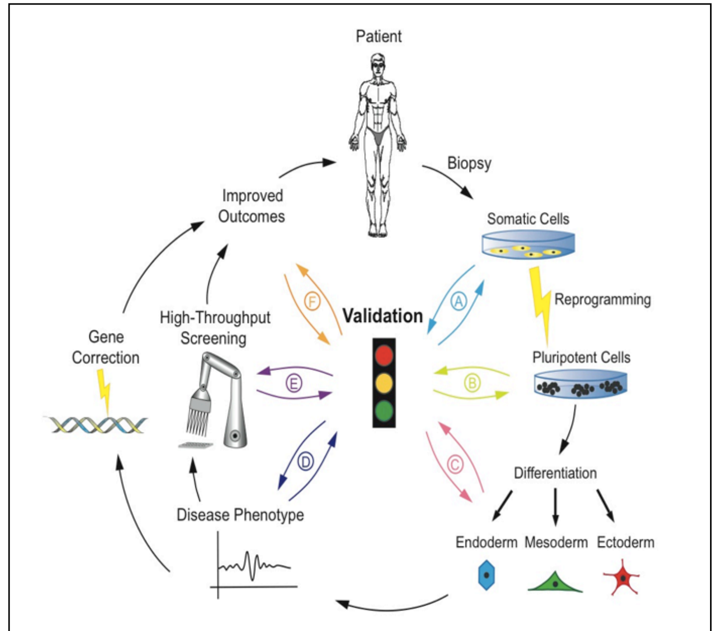

Figure 2: Schematic showing the process of generation, validation, properties and potential uses of human induced pluripotent stem cells both as clinical and basic research tools. (From Siller et al, 2013 [72].

Initially, methods used to generate hiPSCs relied on integrative approaches that utilised either retro- or lentiviral mediated gene transfer. The permanent presence of these exogenous factors in the reprogrammed iPSC lines make them unsuitable for downstream uses such as cellular therapy due to the risk of spontaneous reactivation of oncogenic transgenes [73–75]. However, recent work has demonstrated that integration of the reprogramming factors is not necessary for successful cellular reprogramming (for a complete review of all reprogramming

technologies, see Hu K, (2014) [76]; for an overview of integration-free reprogramming methods, see Zhou, Y and Zeng, F, (2013) [77]). For clean hiPSC production a number of methods have been developed including episomal mediated delivery of reprogramming factors, microRNA (miRNA), recombinant proteins, and non-integrating RNA based Sendai Virus [72, 77]. It is important to note that although these new integration free methodologies should exclude the possibility of any integration events in the somatic genome, this must be confirmed experimentally for all lines. Indeed, in the case of episomal based approaches, a number of the resulting clones (2/7 clones) were found to contain integrated portions of the reprogramming factors, thus rendering them unsuitable for further therapeutic applications [78]. Additionally, there have been reports of hiPSC generation using recombinant proteins fused to cell penetrating peptides, mini-circle DNA plasmids and miRNAs, all of which contribute to the vast array of currently available platforms to generate integration-free hiPSCs [79–83].

Another key issue to be resolved is the extent to which reprogrammed hiPSC fully recapitulate their embryonic counterparts. There is division in the reprogramming field, with some groups suggesting that hiPSCs harbour a higher levels of spontaneous mutations and contain hotspots within the epigenome recalcitrant to complete reprogramming [84, 85]. Initially, it was suspected that the reprogramming process introduced spontaneous mutations into the cells. However, further analysis by other groups have compared greater numbers of hESC vs hiPSC lines that have been derived and cultivated in multiple labs and found a higher degree of variability between laboratories, than between hESCs or hiPSCs [86, 87]. This indicates that methods used to derive, cultivate, cryopreserve and ultimately maintain pluripotent stem cell (PSC) lines has a greater influence upon their quality and functionality than whether they are

from an embryonic or a reprogrammed origin [86]. Clonal analysis of fibroblasts, which are undergoing reprogramming, has demonstrated that mutations present in hiPSC clonal lines were previously present in the initial fibroblast cells rather than being induced as *de novo* mutations via the reprogramming process [174].

From hPSC to Hepatocytes: Follow the Developmental Road

hPSCs, with their capacity of limitless proliferation and tri-lineage differentiation potential, combined with the opportunity to generate hiPSC lines from genetically diverse backgrounds, provides tremendous potential for disease modeling, regenerative medicine and *in vitro* toxicity screening [72]. In order to realise their full potential, hPSCs must be differentiated to the cell type of interest (neurons, cardiomyocytes, hepatocytes, etc). Hepatocyte differentiation is of particular interest to the stem cell field due to the possibility of creating a limitless supply of cells to test novel compounds in terms of hepatotoxicity, as well as disease modeling with patient samples (Fig. 3). In order to coax hPSCs to differentiate into the cell type of interest, it is crucial to understand the

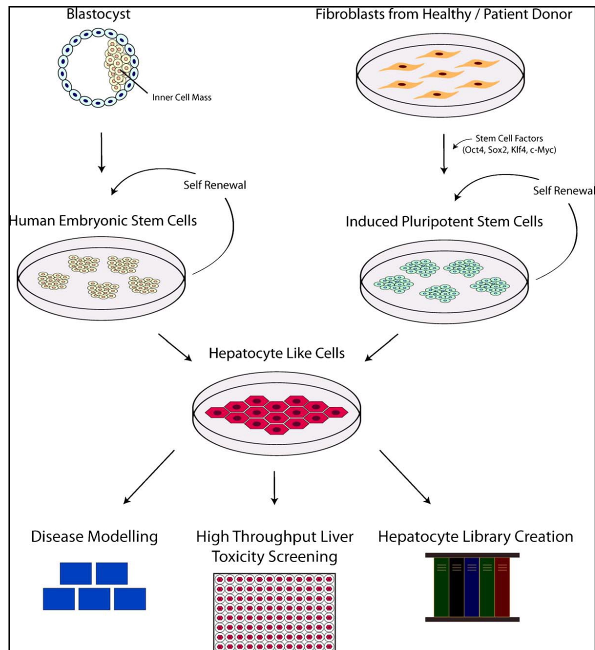

Figure 3. Application of human pluripotent stem cells to in vitro approaches to study hepatotoxicity and basic hepatocyte biology. Taken from Greenhough et al, 2010 [179].

developmental pathways that are responsible for specifying different cell types and organs during in vivo development and organogenesis. Armed with this knowledge, largely gleaned from the study of mouse, Drosophila, and Xenopus as model organisms, the *in vivo* developmental process can be recapitulated *in vitro*.

Endodermal Differentiation: Collaboration between Activin / Nodal, WNT, and BMP

The Activin / Nodal pathway is a crucially important pathway for numerous biological functions *in vivo* including organogenesis and tissue homeostasis [88]. Interestingly, Activin / Nodal signaling is implicated both in the maintenance of self-renewal and pluripotency of hPSCs and mouse epiblast stem cells (mEpiSCs) [89], but has also been widely considered necessary for the directed differentiation of PSCs towards definitive endoderm (DE) in both model organisms such as the mouse as well as in humans [90–93]. Activin A, a member of the Transforming Growth Factor Beta (TGF-β) superfamily of proteins, can be used *in vitro* to induce the expression of *NODAL*. The role of Activin / Nodal in the balance between pluripotency and lineage commitment is complicated, and is thought to be regulated through interactions with other signaling mechanisms, most notably Fibroblast Growth Factor (FGF), Bone Morphogenetic Protein (BMP) and Wingless-Type MMTV Integration Site Family (WNT) / β-Catenin signaling pathways [88]. In hESCs and mEpiSCs, pluripotency and self-renewal are maintained through co-operation between the Activin / Nodal and FGF signaling pathways [94]. Previous reports have shown that FGF receptor inhibition can be compensated by the addition of high levels of exogenous Activin A, however inhibition of Activin A signaling cannot be rescued by increased levels of FGF [95]. Therefore, it is currently believed that FGF signaling does not maintain pluripotency in hESCs alone, but rather acts in collaboration with Activin, potentially through

activation of *SOX2* via Extracellular Signal-Regulated Kinase 2 (ERK2), which is a downstream effector of FGF [88, 96, 97].

In addition to its role in pluripotency, numerous studies have shown that Activin / Nodal signaling is required for DE induction both *in vivo* and *in vitro* [98–100] and this knowledge has led to its ubiquitous use in endodermal differentiation. The critical question is therefore the precise mechanism by which Activin / Nodal can be critical both for maintenance of pluripotency as well as endodermal differentiation. Previous work has demonstrated that the different roles of Activin / Nodal cannot be explained by the levels of signaling present, as providing extra exogenous Activin A did not drive differentiation, but rather maintained a pluripotent stem cell state [95]. The current literature suggests that Activin / Nodal signaling mediates mesendoderm differentiation through interactions with other signaling pathways such as BMP and WNT pathways [101]. Activin A has been shown, when in the presence of high levels of BMP signaling, to repress endodermal differentiation by interacting with key mesodermal markers such as Brachyury (T) [102–104]. However, Activin signaling can also cooperate with the WNT pathway to drive endodermal differentiation through the activation of *NODAL* and Teratocarcinoma-derived Growth Factor 1 *(CRIPTO)* [101]. Ultimately, endodermal differentiation is driven by the downstream Mothers Against Decapentapligic Homolog 2/3 *(SMAD2/3)* complex [105]. This has led to some, such as the Vallier group, to speculate that BMP and WNT are only required to initiate the endodermal differentiation program rather than being key drivers of differentiation [88]. Indeed, BMP blocks the ability of Activin A to enhance self-renewal of hPSCs *in vitro* [106]. On the other hand, WNT and β-catenin induce and interact with the endodermal regulator SRY (Sex Determining Region Y)-Box 17 *(SOX17)*, in coordination

with *Smad2/3* and thus activate Forkhead Box A2 (*FOXA2*) expression [107]. Ultimately the complex regulatory networks that contribute to the exit from pluripotency and commitment to definitive endoderm are not clearly established at present [88].

Hepatic Specification

After exiting the PSC state, and differentiation to DE, the next developmentally relevant stage is commitment to the hepatic lineage. This is coordinated by a host of transcription factors, which are already activated in the DE population, such as *FOXA2*, *SOX17* and Hematopoietically Expressed Homeobox (*HHEX*), as well as key signaling pathways such as BMP4 and FGF4. During hepatic specification, the cells transform into hepatic progenitors, or hepatoblasts, which are bi-potential, meaning they can give rise to either hepatocytes or cholangiocytes. This process involves the proliferation and migration of hepatoblasts out from the primary liver bud and is primarily coordinated through the interaction of Prospero Homeobox 1 *(PROX1)* and T-Box Protein 3 *(TBX3)* [108, 109]. Two of the most critical transcription factors during this stage of development are the pioneer factors *FOXA2* and GATA Binding Protein 4 *(GATA4)* [110, 111]. These two genes are capable of binding to repressed chromatin, which in turn leads to the activation of key hepatocyte markers such as *ALB*. The key pathway that mediates the decision of whether a hepatoblast develops into a hepatocyte or cholangiocyte is the Notch pathway. If Notch is activated in hepatoblasts, they will differentiate to cholangiocytes whereas if Notch is not present, they will form hepatocytes. This knowledge of liver development has now been exploited to mediate the differentiation of hPSCs to cholangiocytes *in vitro* for toxicity screening and disease modelling [112–115].

HLC Differentiation from hPSC: Current State of the Art

The current state-of-the-art with regards to *in vitro* differentiation of hPSCs towards HLCs is exemplified by a number of publications (Fig. 4) [53, 54, 58, 59, 116–120]. These protocols are based on previous work which elucidated the critical developmental pathways in the mouse system [90–93]. These studies demonstrated the critical importance of two primary signaling pathways for directing the initial stage of differentiation from the pluripotent state towards an endodermal fate: namely the WNT and Activin / Nodal pathways. Of these, it has been traditionally been thought that exogenous activation of the Activin / Nodal pathway is critical for endodermal differentiation [88]. However, recent work by Engert and colleagues (2014) demonstrated that WNT signaling is implicated in the induction and maintenance of SOX17 expression in differentiating PSCs [121].

All currently available protocols describing the differentiation of hPSCs towards endoderm lineages are dependent on the use of a variety of exogenously added recombinant growth factors (Fig. 4). This raises a number of issues associated with the use of recombinant growth factors in differentiation. Firstly, recombinant growth factors are extremely costly to produce and verify, in turn severely limiting the amount of hPSC derived HLCs that can be produced. Additionally, growth factor production is notorious for being subject to batch-to-batch variability in terms of potency, making it necessary to verify every batch, further increasing the cost and creating further barriers for scalable production of hPSC-derived HLCs.

<table>
<tr><th>Cell Type</th><th colspan="21">Days in Culture</th><th>Ref.</th></tr>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td><td>13</td><td>14</td><td>15</td><td>16</td><td>17</td><td>18</td><td>19</td><td>20</td><td>21+</td><td></td></tr>
<tr><td rowspan="5">hESC</td><td colspan="7">DMSO</td><td colspan="9">HGF</td><td colspan="4">HGF + OSM</td><td></td><td>122</td></tr>
<tr><td colspan="3">Activin A</td><td colspan="5">FGF4 + BMP4</td><td colspan="5">HGF</td><td colspan="5">OSM + DEX</td><td colspan="3"></td><td>54</td></tr>
<tr><td colspan="5">Activin A + Sodium Butyrate</td><td colspan="7">DMSO</td><td colspan="7">HGF + OSM</td><td colspan="2"></td><td>123</td></tr>
<tr><td colspan="5">Activin A</td><td colspan="6">FGF4 + HGF</td><td colspan="9">FGF4 + HGF + SOM + DEX</td><td></td><td>124</td></tr>
<tr><td colspan="3">Activin A + Wnt3a</td><td colspan="5">DMSO</td><td colspan="9">HGF + OSM</td><td colspan="4"></td><td>60</td></tr>
<tr><td colspan="23"></td></tr>
<tr><td rowspan="6">hiPSC</td><td colspan="3">Activin A</td><td colspan="4">FGF4 + BMP4</td><td colspan="6">HGF + KGF</td><td colspan="8">OSM + DEX</td><td>61</td></tr>
<tr><td colspan="3">Activin A + Wnt3a</td><td colspan="2">Activin A</td><td colspan="4">DMSO</td><td colspan="7">HGF + OSM</td><td colspan="5"></td><td>62</td></tr>
<tr><td colspan="5">Activin A</td><td colspan="5">BMP4 + FGF2 + 4% O_2</td><td colspan="5">HGF + 4% O_2</td><td colspan="4">OSM</td><td colspan="2"></td><td>119</td></tr>
<tr><td>Activin A</td><td colspan="4">Activin A + FGF2</td><td colspan="13">BMP2/4 + FGF1/2/4</td><td colspan="3">HGF + EGF+ OSM + DEX</td><td>125</td></tr>
<tr><td colspan="2">Activin A + FGF2</td><td colspan="3">Activin A + FGF2 + BMP4+ LY294002</td><td colspan="3">FGF10</td><td colspan="2">FGF10 + RA + SB431542</td><td colspan="10">FGF4 + HGF + EGF</td><td></td><td>58</td></tr>
<tr><td colspan="5">Activin A</td><td colspan="5">FGF4 + HGF</td><td colspan="10">FGF4 + HGF + OSM + DEX</td><td></td><td>126</td></tr>
</table>

Matrigel | MEFs | Collagen | FBS / Fibronectin

Figure 4. Diversity in available growth factor based hPSC derived hepatocyte differentiation protocols. DEX= Dexamethasone; HGF= Hepatocyte Growth Factor; KGF=Keratinocyte Growth Factor; RA=Retinoic Acid

A number of protocols that describe growth factor free differentiation to other lineages such as the ectoderm (neurons) [52] and mesoderm (cardiomyocytes) [42] have been reported recently. These protocols use small molecule surrogates, which mimic the respective growth factors and activate the appropriate pathways elucidated by our understanding of developmental biology. However, to date, there have been no reports of any protocol for the differentiation of hPSCs to an endodermal lineage driven entirely by small molecules. Many small molecule mimetics have been developed to target both the WNT and Activin / Nodal pathways due to their critical influence on both embryonic development as well as their roles in cancer development [88].

There have been a number of attempts to improve endodermal differentiation of hPSCs through the use of small molecules. A number of reports have focused on the priming of undifferentiated hPSCs with small molecules, to enhance their ability to produce endoderm, followed by the use of traditional growth factor differentiation methods [127]. Other groups, such as the Melton group focused on screening large libraries of small molecules which could drive endodermal differentiation of hPSCs [128]. The Melton group reported in 2009 that in a screen of 4000 chemicals, two small molecules, namely IDE1 and IDE2 could efficiently direct hPSCs towards definitive endoderm. They reported that these small molecules resulted in approximately 80% of the cell population expressing the DE marker SOX17, a significant improvement over traditional growth factor approaches. However, while these small molecules apparently induced efficient endodermal differentiation to DE, these cells were not further differentiated to endodermal tissues such as hepatocytes or pancreatic cells.

The use of certain WNT mimetics (agonists) such as CHIR99021 (CHIR) and 6-bromoindirubin-3–oxim (BIO), which work through the inhibition of Glycogen Synthase Kinase 3β (GSK3β) which is a component of the β-catenin destruction complex, have been shown to be effective enhancers / primers of endodermal differentiation [124, 125, 122, 126]. Several publications have shown the efficacy of a brief treatment with these inhibitors in directing endodermal fate decisions [132]. However, to date all endoderm differentiation procedures that utilise an initial treatment with GSK3β inhibition, have been followed by treatment with the recombinant growth factor Activin A. Interestingly, the essential control experiment leaving out Activin A to decipher whether WNT signaling alone is sufficient to derive DE formation were not performed [120, 122, 123, 125-127].

With these points of references the data presented in Paper I of this thesis represents a new paradigm, whereby we demonstrate that WNT signaling is found to be sufficient to direct the differentiation of hPSCs to DE and thus provide the starting material for subsequent hepatocyte differentiation. Further differentiation towards hepatic progenitors has been previously optimised using the small molecule dimethyl sulphoxide (DMSO), and thus is already small molecule based [60, 62]. For the final step of the procedure we first identified the minimal requirements for the conversion of hepatic progenitors to HLCs. We identified that Hepatocyte Growth Factor (HGF) and dexamethasone (DEX) were absolutely required, and we therefore searched the literature for small molecule mimetics of HGF, and found an HGF receptor agonist, N-hexanoic-Tyr, Ile-(6) aminohexanoic amide (dihexa) [133]. The presented protocol is composed of 3 phases, each driven by small molecules that mimic the developmental trajectory from hPSCs to HLCs: Phase I (WNT agonist CHIR), Phase II (DMSO), and Phase III (dihexa and DEX).

This resulted in a significant reduction in both cost and variability that is associated with and impedes growth factor based methodologies.

Stem Cell Derived Hepatocytes from Non-human Animal Models

In addition to hPSC derived HLCs, researchers have also leveraged off hiPSC based technologies which are in routine use for modeling disease *in vitro* and transferred this knowledge to other non-human species. An approach being developed and showing great promise is the reprogramming of somatic cells from different species. To date a number of non-human species have been reprogrammed, including mice, rats, canine, pig, horse, non-human primates, as well as a number of endangered species including the northern white rhino [66, 129–134]. This technology, and subsequent directed differentiation, for example to HLCs, could potentially yield more information into interspecies variation in hepatic metabolism and provide a means of selecting the most relevant *in vivo* model for testing various compounds. As previously described, there is interspecies variation in the metabolism of drugs via the CYP450 enzymes, and by first screening species specific PSC-derived HLCs, one could potentially determine which animal model is the most suitable and relevant to human biology. This will not only decrease the cost of screening drugs in both small and large animal models, but will significantly reduce the number of animals required and reduce their suffering during toxicity trials. Therefore the application of PSC differentiation protocols, which derive HLCs fits well into the framework of the 3Rs as discussed previously.

Current Limitations and Future Perspectives

Ultimately, the hepatotoxicity field is moving towards *in vitro* based models for assessing drug safety and efficacy to reduce the cost of drug development and to reduce the number of animals required for safety assessment. There are a number of limitations to the use of *in vitro* hepatocyte models such as cultured PHHs and HLC lines due to their reduced hepatic functional characteristics. The limited availability of PHHs, combined with their short *in vitro* lifespan has lead to the development of immortalized hepatocyte cell lines through genetic manipulation or by isolation of lines from hepatic cancer. However these cells lines do not fully recapitulate all aspects of their *in vivo* liver counterparts. Additionally, these lines are not useful for the study of genetic liver disease or the effects of CYP450 polymorphisms due to their origin from a single donor. A promising solution to these problems is the development of hepatocyte models based on the differentiation of hPSCs into HLCs. hPSCs have the capacity for nearly limitless self-replication *in vitro* and thus vast numbers of HLCs can be derived. However, the differentiation of HLCs from PSCs has been limited by the high cost of the growth factors required to direct their differentiation, as well as variation amongst various hiPSC lines in terms of their differentiation potential. As a consequence, there is great interest in developing cost effective novel approaches for HLC differentiation, which are completely driven by small molecules and devoid of growth factors.

To address these concerns and to improve upon the current state of the art with respect to *in vitro* hepatotoxicity models, this thesis reports three key developments: (i) The development of a novel, small molecule protocol for the differentiation of hPSCs to functional HLCs; (ii) a method for rapidly assessing the capacity of multiple hPSC lines to differentiate into

definitive endoderm, the starting point for HLC differentiation; and (iii) in collaboration with the University of Edinburgh, we report a new non-invasive model of hepatotoxicity in conjunction with the cell line HepaRG, providing a means to assess hepatotoxicity both in real time and at various concentrations of the drug of interest. These three reports will potentially have a significant impact on the field of hepatotoxicity in terms of reducing cost and variation, as well as increasing the sensitivity of *in vitro* hepatotoxicity models.

Key Methodologies

Human Pluripotent Stem Cell Culture

hPSCs (human embryonic and induced pluripotent stem cells) were cultivated under feeder free conditions using growth factor reduced Matrigel (Sigma) coated plates or recombinant human Vitronectin (Life Technologies) coated plates, in Essential 8 media (Life Technologies). Cells were routinely passaged using 0.5 mM Ethylenediaminetetraacetic Acid (EDTA) (Life Technologies) when cells reached 80 - 85% confluency. Cells were maintained in a humidified incubator with 5% CO_2 at 37^oC. To ensure that the cells were pluripotent, all lines were routinely assessed by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and immunofluorescence for expression of the key pluripotency markers OCT4 (POU5F1), NANOG, and SOX2.

Human Induced Pluripotent Stem Cell Generation and Validation

In this study, we have utilised various hiPSC lines that were generated in house. All parental fibroblast lines were purchased from the American Type Culture Collection (ATCC). The lines were as follows: CRL-2097 (fibroblast line derived from a normal newborn human male); BJ (CRL-2522, fibroblast derived from a newborn human male foreskin sample); and Detroit (Detroit 551, CCL-110, human foetal female, fibroblast line). We used both integrative retrovirus and integration-free Sendai virus methods of reprogramming. The retroviral particles carrying the Yamanaka factors *(POU5F1/OCT4, SOX2, KLF4, c-MYC)* were purchased from Vectalys (France) and the reprogramming was performed using a multiplicity of infection (MOI) of 5, as previously described by Vallier and colleagues [140]. For Sendai virus mediated

reprogramming, the CytoTune^{TM}-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) containing the Yamanaka factors was used. Reprogramming was in accordance with the manufacturer's instructions. For both retroviral and Sendai virus based approaches, hiPSC colonies were manually picked into 12 well plates, pre-coated with growth factor reduced Matrigel (Sigma) and supplemented with Essential 8 Medium (Life Technologies). Cells were routinely passaged as described above for human pluripotent stem cells. The resulting hiPSC lines were cultivated and banked routinely. In addition the resulting hiPSCs were thoroughly characterised as follows: (i) RT-qPCR (using hESC line H1 as a control) and immunofluorescence against OCT4/POU5F1, NANOG, and SOX2 expression. (ii) Assessment of pluripotency via directed differentiation to cell types representative of the three embryonic germ layers: mesoderm (beating cardiomyocytes) [43], ectoderm (neurons) [52], and endoderm (hepatocytes) [62]. (iii) For retroviral reprogramming, the hiPSCs were characterized after passage 10 to ensure silencing of the exogenous pluripotency factors via reverse transcription polymerase chain reaction (RT-PCR). (iv) For Sendai reprogramming, the hiPSCs were characterised after passage 10 with TaqMan^{TM} iPSC Sendai Detection Kit (Life Technologies) to demonstrate that the resulting hiPSCs were free of Sendai virus. (v) hPSCs were characterised for a normal karyotype. Karyotyping was performed using the KaryoLite™ BoBs™ assay. The assay uses a panel of probes that recognise the centromeres, p and q arms of chromosomes 1-22, X and Y, and q arms of acrocentric chromosomes. Karyotyping was performed at the Turku Centre of Biotechnology, Finland (http://www.btk.fi/microarray-and-sequencing/services/karyotyping-service/). For the studies described in this thesis, all validated hiPSCs were used and differentiated between passage numbers 25-40.

Human Embryonic Stem Cell Lines

In the studies presented in this thesis, we have used a number of hESC lines. The hESC line H1 was purchased from the WiCell Corporation and has been extensively used in the literature. The hESC lines 207, 360, and 429 were obtained from the Karolinska Institute, Sweden and have been described previously [141]. We have tested our protocols on a number of male and female lines: H1, 207, 360 were derived from male blastocysts, while the 429 was derived from a female blastocyst. hESCs were cultivated and routinely characterised as described above.

Differentiation of hPSCs to Hepatocyte Like Cells

HLCs were differentiated from hPSCs using either a standard growth factor based approach [116, 117] or our novel small molecule driven protocol [142]. For both protocols, cells were passaged onto growth factor reduced Matrigel (Sigma) coated tissue culture plates at a ratio of either 1:3 - 1:4 in Essential 8 Medium (Life Technologies). Each method will be described separately below.

Growth Factor Protocol

24 hours after seeding, the cells were washed with phosphate buffered saline (PBS) without calcium and magnesium (Life Technologies), the media was changed to Roswell Park Memorial Institute Medium 1640 (RPMI 1640) / B27 (both from Life Technologies), supplemented with 100 ng/ml of Activin A (Peprotech), and 50 ng/ml Wnt3A (Peprotech) to produce DE. hESCs were treated with Activin A and Wnt3A for 72 hours, as previously described [60]. Whilst, hiPSCs required an additional 48 hour treatment with Activin A alone to

induce DE, in line with previous reports [62]¹¹⁷. After induction of DE, the cells were specified to a hepatic progenitor fate over 5 days using hepatic specification media composed of Knockout Dubelcco´s Modified Eagle´s Medium (KODMEM), Knockout Serum Replacement (KOSR), Non-Essential Amino Acids (NEAA), Glutamax, β-mercaptoethanol (all Life Technologies), and 1% DMSO (Sigma) in line with previous reports [60, 62]. Hepatic progenitors were then matured into HLCs using a 9 day treatment with Leibovitz 15 (L-15) Media, supplemented with 8.3% foetal bovine serum (FBS) (BioWest), 8.3% Tryptose Phosphate Broth (Sigma), Glutamax (Life Technologies), Ascorbic acid (Sigma), Hydrocortisone (Sigma), and Insulin-Transferrin-Selenium (ITS) (Life Technologies). This base media was further supplemented with HGF (Peprotech), Oncostatin M (OSM) (Peprotech) and DEX (Sigma) as described previously [60, 62]. Cells were analysed between days 19-21 of the growth factor protocol.

Small Molecule Protocol

For the small molecule based approach, 24 hours after seeding, the cells were washed with PBS and then media was changed to RPMI / B27 (Life Technologies) (with or without insulin) supplemented with 3 - 4 µM CHIR (Stemgent). After a 24 hour treatment with the small molecule the media was replaced with RPMI / B27 without any small molecule supplement to derive DE. After a further 24 hours of differentiation, the media was changed to hepatic specification media composed of KODMEM, KOSR, NEAA, Glutamax, β-mercaptoethanol (all Life Technologies), and 1% DMSO (Sigma) in line with previous reports [60, 62]. The medium was exchanged every 48 hours for the next 5 days. The hepatic progenitors were then directed to HLCs with a 10-15 day treatment with a modified formulation of the L-15 media, containing L-

15 base media (Sigma), 8.3% FBS (BioWest), 8.3% Tryptose Phosphate Broth (Sigma), Glutamax (Life Technologies), Ascorbic acid (Sigma), Hydrocortisone (Sigma), ITS (Life Technologies), and 100 nM each of DEX (Sigma), and dihexa (kind gift of Prof Joseph Harding) [128]. HLCs were analysed for their functional characteristics at day 17-20 of the small molecule driven protocol.

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

It is now widely accepted that there is a minimal amount of information that must be provided when reporting quantitative polymerase chain reaction experiments (qRCR) (set out in the *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments (MIQE) guidelines [143]). To this end the following explains our methodologies for cell collection, processing and gene expression analysis. Cells to be analysed by RT-qPCR were harvested by washing once with PBS, then scraped off the dish into PBS. The resulting cell suspension was centrifuged for 1 minute at 10,000 x g at 4°C. The PBS was carefully aspirated and the cell pellet was resuspended in TriZol reagent (Life Technologies). Ribonucleic acid (RNA) was then isolated according to the TriZol manufacturer's instructions. After isopropanol precipitation, the RNA was resuspended in embryo transfer water (Sigma—hereafter referred to as water) and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop). RNA was either frozen immediately at -80°C or converted to complimentary deoxyribonucleic acid (cDNA) as follows, 500 ng of RNA was routinely converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) in a final volume of 20 µL. cDNA synthesis was performed on a Veritas PCR block (Life Technologies) as follows; 15 minutes 56°C, followed by 2 hours at 37°C. The resulting cDNA was diluted with water to a final concentration of 5 ng/µL for all subsequent analysis. After dilution the cDNA was stored at -80°C until required.

For all gene expression analysis, we used commercially available TaqMan^{TM} gene expression assays, combined with TaqMan^{TM} Fast Advanced Master Mix (all from Life Technologies). The details of these assays can be found in the Supplemental Table S1 of Paper I. All RT-qPCR reactions were prepared according to manufacturer's instructions using 5 ng of cDNA as template. The only deviation from the manufacturer's instructions was to reduce the final reaction volume from 20 µl to 15 µl. The probe sequences are proprietary, and as a consequence, Life Technologies would not provide these when queried, but it should be noted that all the probes were fully verified and spanned an intron, with the exception of *SOX2* which contains a single exon.

Cytochrome P450 Activity Assessment

The CYP450s have a critical role in both drug metabolism and hepatotoxicity. An important methodological consideration encountered during assessment of the small molecule derived hepatocyte-like-cells was the utility of commercially available kits for measuring CYP450 activity. The current state-of-the-art assay for assessment of drug metabolism is through mass spectrometry (MS) and high performance liquid chromatography (HPLC) to assess the various metabolites produced during drug processing and metabolism. However, these methods require both financial and technical resources, which were not available to us throughout the current study. We therefore employed widely used commercially available kits to assess CYP450 activity, via CYP450-specific Luciferin reagents for both a rapid and easy readout. These kits are particularly useful as they can provide a rapid assessment of CYP450 activity from live cells within 30 minutes to 4 hours. However, it is important to consider the controls employed as these can dramatically influence the results. For example, some papers

directly compare HLCs to a culture media (containing Luciferin) control, this will provide the so called 'basal' CYP450 activity. A more robust comparison is to benchmark against another cell line such as hESC/hiPSC or fibroblasts to identify any non-specific cellular influences. To this end, we assessed a number of cell types against each CYP450-specific kit (CYP1A2 and CYP3A4) versus a no cell media control. We tested the following cell types: non-irradiated mouse embryonic fibroblasts (MEFs), irradiated MEFs, human fibroblasts (both male and female), undifferentiated hESCs and hiPSCs (4 different lines), and DE cells derived from hESCs and hiPSCs. All of the cell types tested were found to have minimal detectable basal activity as compared to media control. We chose to only show 'basal' activity from undifferentiated hPSCs in our publication based on the above results. In the future, we intend to expand our investigations, utilising more robust methods such as MS / HPLC to interrogate the specific metabolic profiles of our HLCs upon challenge with xenobiotics which are metabolized by CYP450s.

Enzyme-Linked Immunosorbent Assay (ELISA) Based Assessment of Serum Proteins

One of the critical functions of the liver is the production of serum proteins and clotting factors required for human homeostasis. To assess these functions in differentiated HLCs, we employed Enzyme-Linked Immunosorbent Assay (ELISA) based methods. After differentiation to HLCs (day 17 for small molecule, and day 19-21 for growth factor) the cells were incubated for 24 hours in fresh L-15 complete media (as described above) containing DEX, and dihexa (for small molecule HLCs) or OSM and HGF (for growth factor HLCs). The supernatants were collected and either analysed by ELISA immediately or flash frozen on dry ice and stored at -80°C for subsequent analysis. We used specific ELISA kits to assess human ALB (Alpha

Diagnostics), fibronectin (AbCam) and A1AT (AbCam). In all cases a negative control was used, consisting of L-15 complete media with small molecules or growth factors (as described above) incubated for 24 hours at 37°C and 5% CO_2, to eliminate background readings. After collecting the supernatant, the cells were collected for protein quantification for normalisation purposes. All ELISAs were performed according to manufacturer's instructions, and analysed using a Tecan Infinite F200 plate reader (Tecan) measuring absorption at 450 nm. All samples were analysed in triplicate, and results were presented as the mean of 3 independent experiments, with the error bars representing the standard deviation between the 3 experiments.

General Discussion

Paper I: Small Molecule Driven Hepatocyte Differentiation of Human Pluripotent Stem Cells

In the first paper included in this thesis, we report the development of a novel, small molecule driven differentiation protocol for deriving HLCs from both hESCs and hiPSCs. To date, a number of groups have demonstrated relatively efficient hepatocyte differentiation protocols from hPSCs [53,58,60,61,117,119,123,126]. These protocols take advantage of key events, which occur during organogenesis of the liver in the developing embryo, by striving to mimic the developmental trajectory *in vitro,* through the presentation of a sequence of carefully orchestrated developmental cues to the cells. However, all current methods heavily rely on the use of exogenous growth factors to drive the differentiation process. The derived HLCs approximate to a foetal hepatic phenotype [144] and have utility in disease modeling of liver disease such as A1AD [140, 141], Alpers-Huttenlocher Syndrome [147], as well as hepatotoxicity screening [148]. There are a number of drawbacks associated with the use of growth factors. One being the issue of scalability, the ability to produce meaningful amounts of HLCs at a cost that is realistic—this is a major stumbling block for growth factor based approaches. Which is further compounded with the issue of batch-to-batch variation with respect to potency, requiring each lot to be validated. In order to both reduce cost and variability associated with conventional methods of producing HLCs *in vitro*, we sought an alternative strategy to replace the requirement for growth factors, by identifying small molecule surrogates. Ultimately, the use of small molecules to drive the differentiation will potentially facilitate the development of Good Manufacturing Practice (GMP) quality small molecule derived HLCs. Additionally, many

small molecules are already approved by the U.S. Food and Drug Administration (FDA), further easing the translation to GMP quality differentiation protocol.

Utilising key developmental events / pathways known to orchestrate the differentiation towards hepatocytes, we established a differentiation strategy divided into three main phases which recapitulate key development landmarks: Phase I—Definitive endoderm induction; Phase II—Hepatic specification; and Phase III—Hepatocyte like cell maturation. Each of these three phases results in a different cell type, from DE, to hepatic progenitors, and finally to HLCs.

Phase I: Definitive Endoderm Induction

It is well established that both Activin / Nodal and WNT are crucial pathways for the formation of DE, and there is a general consensus that exogenous stimulation of the Nodal pathway through the use of recombinant Activin A is essential for endodermal specification [88, 105]. However, recent literature suggests that WNT signaling is critical for both the induction and maintenance of key endodermal marks such as *SOX17* [121]. We sought to test this hypothesis, by assessing if manipulation of the WNT signaling pathway could drive hPSCs towards DE. When hPSCs were treated with either recombinant Wnt3A protein either alone or in the presence of recombinant Activin A, surprisingly both treatments yielded DE as assessed by FOXA2 and SOX17 staining at high efficiencies (Wnt3A, 80%, Wnt3A / Activin A, 83%). This observation supported our hypothesis that activation of the WNT pathway was sufficient to drive endodermal specification in the absence of exogenous Activin A. We next assessed a number of small molecules, which specifically activate the WNT pathway, for their ability to derive DE. Interestingly, many current protocols use small molecule WNT agonists to improve the

efficiency of endodermal differentiation by priming the cells prior to growth factor based differentiation. However, to date no protocol or publications had tested the effects of using small molecule WNT agonists in the absence of Activin A in the context of hepatic differentiation. Our findings clearly demonstrated that there was no requirement for exogenous addition of Activin A in order to derive DE. We initially screened a panel of candidates that were known agonists of the WNT pathway [122, 127] with varying outcomes. We identified two small molecules, CHIR and BIO, which proved to be highly potent, and efficiently directed hPSCs to DE. The mode of action of these small molecules is well established and they are both known to act through inhibition of GSK3β [129]. For the differentiation of the hESC line H1, we used 3 µM CHIR or 1 µM BIO to induce DE. These concentrations were chosen based on previous reports which had shown them to be effective in priming / enhancement of endodermal differentiation [127].

A critical consideration with any new differentiation procedure is to ensure that the correct developmental route is followed. In this work we monitored the developmental trajectory of the cells through the first 48 hours of the differentiation to DE. We analysed key genes indicative of Primitive Streak (PS) and DE every 4 hours. We observed that, although the kinetics of the small molecule approach was faster than the traditional growth factor based methods (48 hours versus 72 - 120 hours, protocol dependent), the cells still progressed through the correct developmental route. We observed morphological changes within 24 hours from typical flat cells, to a 3D morphology, indicating a transition to PS. These observations were supported by the time course, with the key mesendodermal / PS genes such as Goosecoid (*GSC),* Brachyury *(T),* Forkhead Box A2 *(FOXA2), NODAL* and Mix Paired-Like

Homeobox 1 *(MIXL1)* showing peak up-regulation at 24 hours. Surprisingly, after just 4 hours of treatment with CHIR, *NODAL* showed significant up-regulation, indicating the endogenous Nodal pathway was activated as a downstream effect of the activation of the WNT pathway, without the addition of exogenous Activin A. After 24 hours of directed differentiation via GSK3β inhibition, we removed the small molecule and allowed the cells to undergo default differentiation in the absence of the WNT agonist. The initial 48 hours of differentiation are critical, as further experiments revealed that both higher concentrations of CHIR or longer treatment times were in fact inhibitory to endodermal differentiation. After this additional 24 hours of differentiation, cells destined to be DE migrated out from the PS forming a monolayer of cells with a typical petal-shaped morphology indicative of DE formation. The DE cell population exhibited up-regulation of key markers such as *FOXA2*, *HHEX*, and *SOX17* both at the transcriptional level as assessed by RT-qPCR and the protein level as confirmed by immunohistochemistry. The developmental time course confirmed that the removal of the WNT agonist was crucial, as elevation in *HHEX* and *SOX17* expression levels were only observed in the second half of the time course, after CHIR had been removed.

A critical aspect of any differentiation protocol is its translation to multiple hPSC lines in a robust manner. When we applied the protocol for DE differentiation, which was optimal for H1 (3 μM CHIR in RPMI/B27 containing insulin) to other hPSC lines, it was very inefficient, often yielding little to no DE induction. Therefore each hPSC line required optimisation with respect to cell density, CHIR concentration and base media composition with respect to insulin (+/- insulin). The base media consideration was flagged up based on previous reports demonstrating that insulin signaling is inhibitory to DE formation [105] and this inhibition could be

overcome through the use of the Phosphoinositide 3-Kinase (PI3K) inhibitor LY294002 [149] or the simple omission of insulin in the base media. To this end, each cell line was optimised for the initial cell seeding density, base media composition (presence or absence of insulin), and CHIR concentration (1-10 µM). Remarkably, all the lines we tested showed efficient DE differentiation when treated with 4 µM CHIR in base media without insulin. The need for different concentrations of CHIR might be due to differences in endogenous WNT levels in the starting hPSC population [150]. WNT is known to be a 'pivot point' between self-renewal and differentiation in hPSCs and this may provide a logical explanation for the observed differences in requirements.

Previous work with growth factor based methodologies demonstrated that hiPSC lines required an additional 2 days of exogenous Activin A (a total of 5 days) to efficiently differentiate to DE [62]. In order to demonstrate that the hiPSC derived DE was indeed fully differentiated and that this process followed the expected developmental trajectory, we performed a 48 hour time course. We assessed the hiPSC line Detroit RA using the optimized conditions (4 µM CHIR in RPMI/B27 without insulin) (See Siller et al 2015 Figures 3 and S3 (Paper I) [142]). The time course revealed a very similar pattern of gene expression to that observed in the hESC line H1, confirming that a 24 hour treatment with CHIR followed by CHIR removal was sufficient to drive DE formation in an hiPSC line. Importantly we found that there was no requirement for an extension of the differentiation timeline for hiPSCs as we had previously observed with growth factor based approaches [62].

A major challenge associated with the use of growth factor based procedures is that many cell lines respond very inefficiently to Activin A / Wnt3A. In our hands we tested a

number of different hESC and hiPSC lines with standard Activin A / Wnt3A [60, 62] differentiation. We observed that that the majority of lines were recalcitrant to DE formation. However, when we applied the optimised small molecule protocol to the same lines, we were able to achieve DE differentiation in all lines at similar efficiencies to those obtained in the hESC line H1.

The demonstration that WNT signaling alone, in the absence of exogenous Activin A signaling, was sufficient to derive DE is of great interest in itself, as this runs counter to the established literature [88]. While this paper has not addressed the precise mechanism by which this is occurs, it provides a very promising avenue for future research. The elegance of the system that we have developed is in its simplicity, speed and cost. As mentioned previously, it is very intriguing that *NODAL* is both rapidly and highly up-regulated in just 4 hours after exposure to the GSK3β inhibitor CHIR. We can now use our system to interrogate the interplay between some of the most fundamental regulators of early embryonic development such as the WNT, Nodal and BMP pathways. There are a number of commercially available small molecules that will allow the dissection of these pathways, allowing us to determine with precision where they interact and what effect it has on the developmental trajectory of hPSCs. Ultimately, this may provide us with a clearer perspective of the interplay between these pathways. In addition it will potentially allow us to precisely control early events in the differentiation process allowing us to toggle the system to derive the end stage cell type of interest. This will be of great interest for a number of lines of research including differentiation into the other endodermal cell types, such as beta-cells, intestinal, and lung cells.

Phase II: Hepatic Specification

The next step towards HLCs was to specify the DE population to hepatic progenitors. This has already been developed by several groups including our own, where we have demonstrated that the small molecule DMSO efficiently directs DE to hepatic endoderm [60, 62]. We applied this established regime to the small molecule derived DE population and demonstrated that it gave rise to hepatic progenitors with high efficiency. These hepatic progenitors expressed key markers such as Hepatic Nuclear Factor 4 Alpha (HNF4A) and Alpha Foetoprotein (AFP) both at the transcriptional and protein level. We analysed key developmental regulators of hepatic specification throughout the 5 day hepatic specification step. We observed that the dynamics of both hESCs and hiPSCs activated markers such as *TBX3* occurred in the correct developmental order. In addition, the dynamics of hepatic specification of small molecule versus growth factor derived hepatic progenitors followed a similar pattern of differentiation, in line with the literature describing liver organogenesis [108–110].

At this stage in the differentiation, the hepatic progenitors are potentially bi-potential, meaning that they can undergo specification to the two main liver cell types, namely hepatocytes or cholangiocytes [108, 110]. Although we have not tested this bi-potenitality to date, there are a number of growth factor based protocols available that mediate differentiation to a cholangiocytic fate [112, 113]. The ease and efficiency of producing hepatic progenitors through the described small molecule driven protocol will potentially facilitate the development of a small molecule driven cholangiocyte differentiation procedure. Regardless, the small molecule derived hepatic progenitors can provide a source of cells for modeling choleostatic diseases [151] *in vitro* using currently available methodologies. In addition, the *in vitro* derived cholangiocytes

will have many applications for improving current hepatocyte culture and maturation through the use of existing coculture [152–156] and liver organoid formation platforms [157–159].

Phase III: Hepatic Maturation

To differentiate hepatic progenitors to HLCs, a number of groups have developed diverse protocols that vary both in duration as well as in the exogenous growth factors used [54, 58, 59, 61, 116, 117, 119]. Initially, we sought to use a commercially available media, HepatoZYME-SFM (Life Technologies) to induce hepatocyte maturation from hepatic progenitors. This media has been shown to enhance the function and maintenance of both PHHs as well as hPSC derived hepatocytes by other groups [160]. In an effort to replace the traditional growth factors normally used in this step of the differentiation, we compared the existing protocols to ascertain which components were critical to hepatocyte formation (see fig. 4). We identified that the most commonly used components were the growth factors HGF, OSM, and the glucocorticoid, DEX. We next identified the minimal requirements in terms of these factors to generate functional HLCs. We found that HGF and DEX in combination with a base media was sufficient. To identify a replacement for HGF, we trawled the literature to identify potential small molecule mimetics. As HGF (or scatter factor) is highly expressed in many metastatic cancers, the majority of effort has been to identify HGF antagonists [161]. While there are numerous HGF inhibitors, both in the literature and commercially available, there was a significant dearth of agonists of HGF. It wasn´t until the start of 2013, when a publication from the Harding lab described an extremely potent HGF activator, dihexa, for the treatment of neurological disorders such as dementia [133]. We investigated if dihexa could act as a surrogate for HGF in our system. We first optimized the concentrations the small molecules dihexa and DEX in the proprietary HepatoZYME-SFM base

media. We demonstrated that 100 nM for both small molecules gave robust differentiation to HLCs, as confirmed by gene expression and immunofluorescence for critical hepatocyte specific markers including ALB, A1AT, HNF4A, and AFP.

After further investigation into the formulation of the HepatoZYME-SFM media, it transpired that Epidermal Growth Factor (EGF) was one of the components [162]. Since we sought to develop a protocol devoid of any exogenous growth factors, HepatoZYME-SFM was no longer a suitable base media for the hepatic maturation step of the protocol. After trying several media formulations with DEX and dihexa, including William´s Meium E, which has been used by numerous groups for hepatocyte maintenance and culture, we finally settled on a modified version of the Leibovitz 15 media (L-15), as previously reported [60, 62]. Using the L-15 base media as described previously, and replacing the growth factors with DEX and dihexa at the optimised concentrations, we were able to efficiently drive hepatic progenitors to HLCs.

The small molecule derived HLCs exhibited a very similar expression profile of key hepatocyte markers when compared to growth factor derived HLCs. It is important to note that in both cases, the differentiated cells expressed higher levels of *AFP*, and lower levels of *CYP3A4* compared to both foetal and adult hepatocytes as assessed by RT-qPCR, which is indicative of a more foetal phenotype in terms of function and gene expression [144]. This is a major challenge for the majority of cell types to date differentiated from hPSCs *in vitro*. While there are indications that culturing the cells in 3D can enhance their maturity and function [157–159], the optimal methods for achieving a fully mature phenotype have yet to be determined. This is an area that will be heavily investigated in the future.

Although the HLCs produced using our small molecule approach, as well as all current growth factor based procedures, exhibit a foetal phenotype, they do however demonstrate a number of key hepatic functions. These include the production and secretion of serum proteins including ALB, A1AT, fibronectin, and fibrinogen. In addition they have the ability to synthesise and store glycogen, and are able to uptake indocyanine green. The levels of these activities in the small molecule derived HLCs were comparable to growth factor derived HLCs.

As discussed in the introduction, a promising application of hPSC derived HLCs is their use in drug toxicity testing for the pharmaceutical industry. In order for these cells to be applied towards the goal of enhancing toxicity testing, it is critical that the drug metabolising enzymes (CYP450) are active and inducible upon challenge with known pharmaceutical products. While both growth factor and small molecule derived hepatocytes are foetal in phenotype, they do demonstrate significant basal and inducible CYP450 activity. We tested two of the most relevant CYP450s that are of interest to the pharmaceutical industry, namely CYP1A2 and CYP3A4. We assessed these CYP450s in terms of their basal activity and their ability to be induced via known inducers. Small molecule and growth factor derived HLCs exhibited similar basal levels of CYP activity. When challenged with the CYP inducers, Omeprazole (CYP1A2 inducer) or Rifampicin (CYP3A4 inducer), we observed a strong induction of the respective CYP450s. This is a very promising result indicating the potential utility of these cells in hepatotoxicity testing with the potential to attract collaborations with industrial partners.

An important caveat in this study, is that the hPSC derived HLCs were not functionally benchmarked against PHHs. The overriding reason for this was due to the high cost of obtaining PHHs, but also due to the limitations of using cryopreserved PHHs, including inter-

donor variability, and rapid de-differentiation upon isolation and re-plating, as discussed above. For these reasons, we felt it was sufficient to compare the small molecule driven protocol to the current gold standard of growth factor derived HLCs. Another important caveat is that this protocol cannot be considered 'fully defined' due to the use of complex reagents, such as the use of an undefined ECM, Matrigel, throughout the protocol, and FBS in the final step of the hepatocyte maturation. These have been identified as limitations for the translation of this protocol towards clinical applications. Ultimately, this paper was not intended to demonstrate a fully defined protocol for the derivation of hepatocytes with comparable maturity and function to *in vivo* or freshly isolated adult hepatocytes. Rather, it is an important step to significantly reduce the cost and variability associated with traditional growth factor based methodologies.

Based on the findings in this paper, a number of interesting research avenues will be pursued. One of the more intriguing aspects of this work was the finding that WNT alone can drive differentiation to DE. We have assessed the derived DE using a battery of endodermal markers, and proven that the cells are in fact of DE origin and not extra-embryonic in nature. A more thorough comparison between WNT-induced DE and Activin A and Nodal induced DE will be very interesting. An interesting publication analysed the developmental competence of Activin A induced DE versus Nodal induced DE [163]. They found that although there are no appreciable differences between the two cell populations on a global transcriptional level, however there were significant differences in developmental competence of the two cell populations upon implantation into developing embryos. Notably, Activin A derived DE contributed much less to the developing gut tube than did Nodal derived DE. This reduced

competence of the Activin A derived DE could not be rescued by prolonged differentiation or by co-treatment with Wnt3A, suggesting that exogenous Activin A treatment of cells *in vitro* is in fact not an exact mimic of the situation encountered *in vivo* during natural embryonic development. Based on the fact that we observed high levels of *NODAL* activation within 4 hours of GSK3β inhibition and the expression was maintained throughout the DE stage, it will be interesting to analyse the developmental competence of our small molecule derived DE and compare it to existing growth factor based protocols. While the gold standard for this assessment would be implantation into developing embryos, as a preliminary step, it would be very informative to assess the *in vitro* differentiation potential of the small molecule DE into other endodermal cell types such as lung, intestine, and pancreas using existing protocols.

Further development and refinement of the small molecule differentiation protocol will be highly beneficial to the field. A key area of refinement is to develop a completely defined protocol with respect to the hepatocyte maturation step. As mentioned previously, our protocol is not fully defined due to the use of undefined reagents such as FBS and Matrigel. We have already shown that the protocol can be performed successfully on recombinant human Vitronectin, which is fully defined. The next step will be to optimise the media composition in the final stage to remove FBS, creating a more streamlined differentiation protocol, capable of fulfilling GMP quality standards.

There are immense efforts to create fully mature terminally differentiated cell types *in vitro*. As previously stated, our differentiated HLCs do not currently recapitulate the full functions of mature hepatocytes. We are now actively pursuing a number of avenues to obtain this, notably through the encapsulation of HLCs in hydrogel based systems to create 3D

structures. Using 3D culture systems, hepatocytes will be seeded with other supportive cell types such as mesenchymal stem cells (MSCs), liver sinusoidal endothelial cells (LSECs), and cholangiocytes, to create organoids that mimic the structure of the liver. This has great potential to enhance hepatic function and therefore will contribute to downstream applications such as bioartificial liver devices, drug hepatotoxicity screening and disease modeling.

Paper 2: Development of a rapid screen for the endodermal differentiation potential of human pluripotent stem cell lines.

As previously described, one of the key challenges to fully realise the potential of hPSCs both for clinical and industrial applications is the ability to generate a representative library of cell lines from diverse genetic and phenotypic backgrounds and subsequently differentiate them to the cell type of interest in large numbers. Recent developments in reprogramming techniques, cell culture compositions and handling apparatuses, as well as automated cell pickers have made the cellular reprogramming process amenable to a high throughput format. However, with large numbers of cell lines being produced, there is a fundamental challenge still remaining: inter-line heterogeneity both in terms of quality and differentiation potential. As a result of this heterogeneity, not all hPSC lines though fully verified and characterised, will be able to undergo differentiation to the required cell type. This is particularly challenging in the endodermal field. To this end, a number of endodermal screens have been devised to gain insight into whether a particular cell line is capable of generating endoderm. Jiang and colleagues (2014) demonstrated that endogenous levels of *WNT3A* expression varied across numerous hPSC lines before differentiation [150]. They observed a correlation between *WNT3A* levels and the ability to produce endoderm. However, in our experience differences in *WNT3A* levels was not an adequate measure of a cell line's endodermal potential. This may be due to the fact that we initially screened the lines and attempted to differentiate them using the growth factor based methodology [60, 62]. Another group led by David Hay and colleagues demonstrated that the expression of AFP in spontaneously differentiated EBs was a useful indicator of a cell line's ability to differentiate to endoderm [164]. However, this approach is

rather time consuming, requiring 2 weeks of differentiation, followed by further processing of the resultant EBs for immunohistochemistry. The read out of this assay was the assessment of the endodermal marker AFP, with the highest expressing EBs deemed the best candidates. There are several key limitations to this approach, including the inherent variability in EB formation, further processing of the EBs for marker visualisation, and the time that this assay takes to perform.

With the report of a small molecule driven endoderm differentiation protocol, we sought to translate these findings into an easy to use, rapid, efficient, robust and cost effective method to assess the endodermal potential of multiple hPSC lines. As previously described, we found that different cell lines had distinct requirements in order to generate DE. To reiterate, we observed a marked difference in differentiation potential with respect to either the presence or the absence of insulin, used to supplement the RPMI base media. Interestingly, we observed a small molecule (CHIR) concentration dependence within a narrow window, which may be a reflection of endogenous *WNT3A* levels (as described previously). The initial screen we performed on multiple hPSC lines was against varying concentrations CHIR (1-10 µM) in base media with or without insulin. We observed that all lines responded to CHIR in a narrow range, between 3 - 4 µM as assessed by DE markers. Intriguingly lower concentrations yielded little to no differentiation and this was reflected in very low levels of induction of key endodermal genes relative to vehicle controls. We found that higher concentrations tended to induce the production of cells of a more mesodermal phenotype, in line with other reports which use 8 – 12 µM CHIR during cardiomyocyte differentiation [42]. This finding allowed us to restrict our DE screen to 4 conditions: 3 µM CHIR, +/- insulin; 4 µM CHIR, +/- insulin.

Using these conditions, we screened 10 different hPSC lines, and were able to accurately predict within 48 hours, purely by cellular morphology, which were suitable for further differentiation to HLCs. In order to confirm the differentiation to DE we used the hESC line H1 as a base line, as we had demonstrated its highly efficient hepatic potential and performed an in depth characterisation of its progeny [142]. In order to corroborate the above screen we performed gene expression analysis and immunofluorescence to validate that the derived cells were indeed DE. After an in depth validation of H1, we reduced the screen parameters to a select number of gene targets which are accepted markers of DE namely *SOX17, FOXA2, HHEX* and *CER1*. For immunohistochemistry, we analyzed SOX17 as the key DE marker to further reduce cost and the time required for validation of DE screen.

While we validated the DE screen across multiple lines using RT-qPCR and immunohistochemistry, it is important to note that this DE screen has no requirement for downstream processing. The ability to simply screen based on morphology within a 48 hour time frame will potentially allow large number of hPSCs to be screened for their endodermal potential. This screen will greatly reduce both the time and cost associated with conventional methods which require RT-qPCR and immunofluorescence.

As a further validation of the screen, we chose 4 hPSC lines to assess their capacity to differentiate beyond the DE stage into hepatocytes. For this assay, we specifically chose 3 lines that passed the initial DE screen based on morphology, RT-qPCR and immunohistochemistry, and 1 line that failed the DE screen. After completion of the small molecule hepatocyte differentiation protocol (as described in Paper I), the cells were analysed based on morphology, RT-qPCR and immunohistochemistry against hepatic markers as described in Paper I. As

expected only the 3 lines which passed the DE screen were capable of terminal hepatocyte differentiation. These three lines demonstrated efficient and robust differentiation as validated by strong immunostaining for ALB and HNF4A as well as induction of key hepatic genes by RT-qPCR. The cell line which was not capable of forming DE in the screen, was found to only marginally up-regulate hepatic genes and presented with low expression of albumin and no expression of HNF4A as assessed by immunohistochemistry.

The above strategy for endodermal potential has been applied to a large number of recently derived hiPSC lines generated from both patient and control cell lines and we have demonstrated that the majority of these lines are proficient to generate functional HLCs using the procedure outlined in Paper 1. As the production of DE is the defining stage/rate limiting step of whether an hPSC can be directed to HLCs (or indeed any endodermal lineage), simply subjecting these cells to just 4 different conditions without having to go through the entire 19 day protocol is a major benefit to the stem cell field in terms of efficiency and cost. In addition, this procedure could easily be translated to an automated high throughput (HTP) format using morphological phenotypical evaluation software such as CellProfiler [165]. In addition, the DE screen will be useful for screening hPSCs for their differentiation potential to a number of other cell types derived from DE, such as lung, pancreas, and gut/intestine cells.

Paper 3: A human HepaRG-based liver impedance biochip reveals acetaminophen-induced disruption of tight junction and cell-substrate adhesions: An enabling tool for drug hepatotoxicity screening.

As previously mentioned, a major limitation to current *in vitro* models of hepatotoxicity is the choice of suitable cell lines that are fit for purpose, as well as the inability to perform longitudinal and physiologically relevant drug testing. In a close collaboration with colleagues at the University of Edinburgh we aimed to develop a "liver-on-a-chip" model to enable a non-invasive and unbiased assessment of structural elements involved in the maintenance of tissue cohesion and polarity. This platform provides a novel approach for drug discovery, disease modeling, and strategies for screening tight junction (TJ) stabilising compounds. To achieve this, we synergistically combined an established model of hepatotoxicity, the cell line, HepaRG [37, 39], with established methods for monitoring electrical impedance of cells in real time in order to study their behavior and response to toxic insult [166–171].

Acetominophen (APAP) is one of the leading causes of DILI [172]. As such there is great interest in the development of robust assays to characterise and quantify DILI and to develop / test relevant small molecules to ameliorate its effects. However, there are a number of problems associated with the development of these assays. As previously discussed, animal models often do not recapitulate the physiological conditions of humans. Additionally, specifically for APAP, there are significant differences with respect to its metabolism between rats, mice and humans [173]. The primary cause of APAP induced hepatotoxicity is the formation of the highly reactive intermediate N-acetyl-p-benzo-quinone imine (NAPQI), through CYP3A4 metabolism [169, 170]. NAPQI causes adenosine triphosphate (ATP) and glutathione depletion, which in turn cause oxidative stress and mitochondrial dysfunction, ultimately leading to cell

death. Hepatocyte function is critically maintained *in vivo* through close cell-cell and cell-ECM contact. The control and maintenance of cell-cell, and cell-ECM contact and indeed polarity is crucially one of the primary reasons isolated PHHs lose function rapidly after perfusion and subsequent *in vitro* culture. One of the most important structures that enables the cell-cell contact are the TJ between cells. TJ are critical for the establishment of cellular polarity and for bile secretion, as well as the expression and localization of drug transporters and CYP450 enzymes [176].

While the detrimental effects of APAP on *in vitro* hepatic models like HepaRG has been reported previously through the use of serum from effected patients [177], this assessment was an endpoint readout, requiring the destruction of the cells for analysis, which prevents longitudinal, and/or real time observation of the hepatotoxic effect. In order to overcome these shortcomings, we have employed an impedance-based cellular assay (IBCA) to monitor the effects of APAP toxicity in real time and in a quantitative manner.

IBCA based methodologies allow for the cells to be grown on a tissue culture dishes which have integrated array of electrodes. These electrodes allow for continual, real time monitoring of various cellular parameters such as cell adhesion, cytoskeletal structural changes and morphological changes of the cells [178]. The principle of IBCA is that small changes in the flow of an electrical current through / across cells occurs when the cytoskeletal structure or the cell adhesion changes in cells which are grown in a monolayer on the bed of electrodes. One of the major benefits of this technology is that it can be monitored in real time, without the requirement for the cells to be sacrificed, nor does it require downstream processing, for example through immunohistochemistry or western blotting to assess the biological effects.

In order to study APAP toxicity in a controlled and real time manner, we chose to use the HepaRG line, due to its high expression / inducibility of CYP450s, as well as its bi-potential nature. We combined this cell line with the IBCA platform technology. After culturing the HepaRG cells for 8 days to attain a mature and terminally differentiated population of HLCs and cholangiocytes. As previously stated, NAPQI is a toxic intermediate that is formed during CYP3A4 mediated metabolism of APAP. Therefore, we treated the cells for 24 hours with the potent CYP3A4 inducer rifampicin, followed by a dose response assay with varying concentrations of APAP.

Our IBCA / HepaRG platform revealed a novel aspect of APAP toxicity. We demonstrated that even at low levels of APAP (sub-toxic), there was a significant dose-dependent disruption of TJs as well as a loss in cell-substrate adhesion. This effect was detectable using the IBCA platform at an early time point (less than 2 hours after exposing the cells to APAP). To confirm these findings, extensive validation was performed using more traditional techniques. The TJs were observed using electron microscopy; using this approach perturbations in TJ integrity was clearly observed. Also, we found perturbations in the localisation / integrity of the TJ proteins using ZO-1, the junctional-associated protein, E-cadherin and cell-substrate proteins (integrins) via confocal microscopy. Importantly, these perturbations were observed at sub-toxic levels in a dose-dependent manner.

We have identified a previously unobserved hepatotoxic effect of treatment with APAP. At sub-toxic levels of APAP, the cell line HepaRG displayed disruption of TJs and cellular adhesions. This novel application of electrical impedance measurement allows for a non-invasive way to monitor these toxic effects in real time, across different concentrations and

time points, thus providing a novel, powerful method for longitudinal toxicity screening non-invasively.

This paper was a truly collaborative study that leveraged off a large team with established methodologies that could be tapped into in order to ensure both high quality and reproducible data sets. For our part we investigated if the observed changes in impedance parameters, notably Rb; z-alpha and Cm, were indeed correlates of epithelial polarization / TJ integrity by conventional methodologies. These included the culturing and differentiation of HepaRG, to obtain a differentiated population of cholangiocytes and hepatocytes. We treated the cells first with rifampicin, which is a potent inducer of CYP3A4. As mentioned above, CYP3A4 is the primary drug metabolising enzyme responsible for the clearance of APAP and importantly for the generation of the toxic intermediate NAPQI. In order to assess whether the observed impedance differences where a consequence of perturbation of TJs, even at levels that are not toxic, the rifampicin treated cells were subsequently treated with different concentrations of APAP. After APAP treatment, we analysed the distribution of ZO-1 by immunohistochemistry.

TJ dysfunction is known to have a critical role in a number of diverse diseases affecting the liver (hepatitis, biliary cirrhosis), inflammatory bowel disease, as well as cancer [175]. Unfortunately, current methods available to study the disruption of TJs *in vitro* require the destruction of the cellular sample for analysis, which impedes the ability to perform longitudinal studies. In addition, this limits our ability to study the effects of known toxins at various concentrations in the same cellular context. The use of electrical impedance modeling

can greatly enhance these studies as data is provided in real time and importantly, cells can be monitored without their destruction during the study.

Using electrical impedance measurements our colleagues were able to reveal a dosage- and time- dependent response to APAP treatment in the hepatotypic cell line HepaRG. At high dosages, the well-established toxicity was observed, as expected. However, at lower, sub-toxic concentrations, significant deleterious effects where observed with respect to membrane and TJ integrity. This was observed first on the novel electrical impedance platform, and confirmed through the use of electron microscopy and immunostaining of the TJ associated protein ZO-1. In control cells, without APAP treatment, ZO-1 staining was clearly observed at the junctions between the HLCs in the HepaRG culture. At high APAP concentrations (20 mM), a significant proportion of the cells detatched from the culture dish, and as a consequence exhibited minimal ZO-1 staining. Importantly, at low, sub-toxic doses of APAP, ZO-1 staining was observed to be highly perturbed, appearing as gaps. This correlated with the data generated by IBCA.

Ultimately this platform will provide a powerful tool for *in vitro* monitoring of the global health and status of hepatocytes over multiple drug treatments. By providing a platform which the non-invasive monitoring of cells, allowing the long term effects of potential drugs to be tested / assessed at various time points and at numerous concentrations.

Conclusions

In this thesis 3 papers have been discussed which can potentially contribute to the development of robust and cost effective *in vitro* models for hepatotoxicity. There are still hurdles that remain before we fully realise and implement these on a large scale for industrial / pharmaceutical interest groups. In the first paper, we described a completely small molecule driven protocol for the differentiation to HLCs from hPSCs. This represents a significant contribution to the field with respect to cost reduction compared with current growth factor based protocols. However, the HLCs generated using the described small molecule approach are not yet of the same maturity and quality as PHHs, as are HLCs generated using growth factors. Furthermore, they suffer the same drawbacks in terms of lifespan as PHHs, significantly limiting their utility in drug development and screening. There are now a number of potential avenues of research that can be undertaken to address these concerns. The ability to generate hepatocytes at a reasonable cost and in a robust manner will facilitate the screening of large libraries of small molecules to enhance both maturity and maintenance of the hepatic phenotype. Ideally, the field requires fully functional hepatocytes that recapitulate the hallmarks of PHHs, whilst allowing long-term maintenance for drug testing. Furthermore, due to the significant reduction in cost, we can now translate our protocol to higher yield platforms such as bioreactors and allowing the generation of large numbers of hepatocytes with relative ease of use. This will significantly contribute to the development of drug screens, by enabling one batch of hepatocytes to be used for many assays.

Additionally, one of the more interesting things that our system allows is the creation of whole liver organoids from differentiated cells. The hepatoblasts that we generate at the end

of Phase II of the protocol are bipotential and can give rise to the two main components of the liver, hepatocytes and cholangiocytes. In combination with other cell types such as endothelial cells and mesenchymal stem cells, it will be possible to form 3D liver organoids. These organoids would essentially recapitulate the architecture of the liver in a miniature, *in vitro* format. The potential for drug screening and disease modeling using this type of system are immense.

In Paper II, we described the development of a rapid, cost effective screen for assessing the endodermal potential of multiple hPSC lines. This is a critical aspect that needs to be addressed and assessed for every new hPSC line generated. With this screen, we now have the potential to rapidly identify from hundreds of hPSC lines the ones capable of generating our tissue of interest. This will be especially useful in the creation of biobanks for tissue transplantation as well as for *in vitro* screen development. One interesting application of this screen will be its potential to contribute to the genome editing field. With the many emerging genome editing technologies coming online, there will be myriad of genetically modified lines created. As a consequence, each clone must be individually verified for pluripotency as well as its capacity to differentiate into the cell type of interest.

Finally, in paper III we have presented a novel platform based on impedance measurements of cell cultures to study APAP hepatotoxicity. This platform has great potential to be used to assess other drugs, due to its robust and quantitative nature, as well as the fact that the cells are not destroyed during the assay. Having real time measurements of hepatocyte cellular health will be of tremendous value as this will facilitate the study of drug metabolism over longer periods of time without the destruction of the cells. Ultimately by

combining this platform with hPSC derived HLCs, the functional applications towards disease modeling and toxicity screening are very promising. This will lead towards the realisation of truly personalised medicine, whereby cells of a patient are differentiated to HLCs, allowing the pre-screening *in vitro* of candidate drugs before the patient receives them.

Ultimately the cost-effective hepatocyte differentiation protocol, presented in Paper I, can be applied to numerous hPSC lines identified to be proficient in endoderm potential, as presented in Paper II, and incorporated into the enabling technology platform presented in Paper III, thus substantially improving current *in vitro* models of hepatotoxicity. There are many technical and biological issues which remain unresolved before these technologies can be successfully implemented in an industrial or clinical setting, including a thorough analysis of the hPSC derived HLCs in terms their maturity and function compared to PHHs. These include improved protocols for the maturation and maintenance of hPSC derived HLCs, as well as the verification of their use for a broad spectrum of drug toxicity assays. With these limitations in mind, the work presented in this thesis will enable many laboratories to produce HLCs in sufficient quantities, at a reasonable and affordable cost, allowing further refinement and hopefully broader applications and improvement of *in vitro* hepatocyte models.

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