Liver cell types derived from pluripotent stem cells

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences.

2014

Sean Harrison
School of medicine
Faculty of Medical and Human Sciences
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List of publications arising from this thesis


List of presentations arising from this thesis

May 2013
Poster at the EMBO workshop on liver and pancreas development, function and disease. 
Differentiation of hESCs to cholangiocyte-like cells in a 3D culture system.

June 2014
Presentation at the BASL basic science retreat.
Hepatic organoid differentiation from human embryonic stem cells
List of abbreviations

αSMA……………..α-Smooth Muscle Actin
AA………………..Actin A
AAT……………..α1-antitrypsin
ABT…………….. Aminobenzotriazole
ADR……………… adverse drug reaction
AFP……………. α-fetoprotein
AGM……………. Aorta-gonad-mesonephros
aHSC…………… activated HSC
ALB…………….Albumin
ALCAM…………..Activated Leukocyte Cell Adhesion Molecule
ALI3D………….Air Liquid Interface 3D
ALP…………….Alkaline Phosphatase
ALT…………….Alanine Transaminase
APC…………… Adenomatosis Polyposis Coli
ASGPR………… Asialoglycoprotein Receptor
AST…………….. Aspartate Transaminase
BEC…………….Biliary Epithelial Cell
BDL…………… Bile Duct Ligation
BMP…………... Bone Morphogenic Protein
BrdU…………….5-Bromo-2’-Deoxyuridine
BSA…………… Bovine Serum Albumin
C…………………Collagen (Table 1-2 only)
CEBP………….. CCAAT/Enhancer Binding Protein
CBLD…………..Chronic Biliary Liver Disease
CD……………… Cluster of differentiation
CK……………… Cytokeratin
CLC…………….Cholangiocyte-like Cell
COL…………….Collagen
CPS1………….. Carbamoyl phosphate synthase 1
CS……………… Carnegie Stage
CYP…………….Cytochrome P450
CYPOR…………..Cytochrome P450 oxidoreductase
DAB……………..3,3’-Diaminobenzidine
DAPM…………..4,4’-Methylenedianiline (4,4’-diaminodiphenylmethane)
DDC…………..Diethoxycarbonyl Dihydrocollidine
DE……………..Definitive Endoderm
DES…………..Desmin
DEX…………..Dexamethasone
DILI…………..Drug Induced Liver Injury
DLS…………..Duct-like Structure
DMSO…………..Dimethylsulphoxide
E……………..Embryonic Day
EB…………..Embryoid Body
EC…………..Endothelial Cell
ECAD…………..Epithelial Cadherin
ECM…………..Extracellular Matrix
EGF…………..Epidermal Growth Factor
EGFP…………..Enhanced GFP
EHBD…………..Extra Hepatic Biliary Duct
ELISA…………..Enzyme-Linked Immunosorbent Assay
EMT…………..Epithelial Mesenchymal Transition
EPCAM…………..Epithelial Cell Adhesion Molecule
ER…………..Endoplasmic Reticular
ESC…………..Embryonic stem cell
EST…………..Expressed Sequence Tag
FACS…………..Fluorescence-Activated Cell Sorting
FBS…………..Foetal Bovine Serum
FGF…………..Fibroblast Growth Factor
FLK…………..Fetal Liver Kinase
FOX…………..Forkhead Box
GFAP…………..Glial Fibrillary Acidic Protein
GFP…………..Green Fluorescent Protein
GGT…………..γ-Glutamyl Transferase
GS…………..Glutamine Synthetase
GSTp.................Glutathione S transferase π
HCM................Hepatocyte Culture Medium
HES1.................Hairy and Enhancer of Split 1
HLC..................Hepatocyte-like Cell
HNF..................Hepatocyte Nuclear Factor
HRP..................Horseradish Peroxidase
HSCs................Hepatic Stellate Cells
HSP47..............Heat Shock Protein 47
HUVEC.............Human Umbilical Vein Endothelial Cells
ICC..................Immunocytochemistry
ICG..................Indocyanine Green
IgG..................Immunoglobulin G
IL-6................Interleukin-6
IHBD.................Intra Hepatic Biliary Duct
iHSC.................inactive HSC
iTRAQ...............isobaric tagging for relative and absolute quantification
ITS..................Insulin-Transferrin-Selenium
JAG..................Jagged
KGF..................Keratinocyte growth factor
KO....................Knockout
L......................Laminin (Table 1-2 only)
LAM..................Laminin
LDL..................Low Density Lipoprotein
LFT..................Liver Function Test
LHX2.................LIM/homeobox protein 2
LKO..................Liver KO
LSEC.................Liver Sinusoid Endothelial Cell
LYVE1..............Lymphatic Vessel Endothelial Hyaluronan Receptor 1
M.................Matrigel (Table 1-2 only)
MC..................Mesothelial Cell
MEF...............Mouse Embryonic Fibroblasts
MESP1.............Mesoderm Posterior 1
miRNA.............micro RNA
TAT…………….Tyrosine Aminotransferase
TGFβ……………Transforming Growth Factor β
TGFBR2………TGFβ Receptor 2
VEGF…………..Vascular endothelial growth factor
VEGFR…………VEGF Receptor
VKO……………VEGF KO
WB……………..Western blot.
wpc……………Weeks Post Conception
WT…………….Wild Type
WT1……………Wilms Tumor 1
Abstract

Liver development involves the differentiation and interaction of both endoderm and mesoderm cell types. The role of the liver in drug metabolism makes it an important area of medical research. Mimicking embryonic liver development in vitro using human ESCs is a strategy used to differentiate liver cell types. These can then be used as a model playing a role in the development of drugs and the study of their hepatotoxicity and would also have potential for use in cell therapy and regenerative medicine.

Differentiated hepatocyte-like cells were found to have more in common with liver cells than those of other organs, including the secretion of albumin and activity of proteins important in drug metabolism, CYP3A and CYP2D6. However the hepatocyte-like cells were found to more closely resemble fetal rather than adult hepatocytes.

Organoid differentiation resulted in cells types which in vivo are both endoderm and mesoderm derived cells of the liver. Culture in this 3D system allowed the spontaneous acquisition of polarity by these cells and their formation into structures reminiscent of liver architecture. After treatment with the toxin 4,4’-diaminodiphenylmethane a cell type and structure specific dose response was observed which matches that described in vivo.
Declaration

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1 Introduction

The overall aims and objectives were to derive hepatic cells from hESCs by mimicking in vitro human development which can then be used to model hepatotoxicity.

Section 1.1 contains the review “Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening” which describes the need for the development of better in vitro models for hepatotoxicity and how the differentiation of hepatocyte-like cells (HLC) from pluripotent stem cells (PSC) can meet this need. This is followed by a more in depth look at the development of the liver (1.2) and its constituent cell types. Section 2 is a research paper describing the differentiation of human embryonic stem cells (ESC) to HLCs by mimicking in vivo development with particular attention paid to characterising their drug metabolising capabilities. Which comes to the conclusion that these HLCs and those derived by others more closely resemble fetal than adult hepatocytes. The last research paper style chapter (3) shows currently unpublished work which derives hepatic organoids containing multiple liver cell types from human ESCs, and shows their use modelling hepatic injury involving multiple cell types.

The review had multiple authors my specific contribution was the drafting of the section “Differentiating pluripotent stem cells towards hepatocyte-like cells” and the design and annotation of figure 4 and 5. My contribution to the paper “Stem cell-derived hepatocyte-like cells mimic fetal rather than adult hepatocytes by transcriptomic, proteomic and functional analyses” was the growth and maintenance of all the hESC and their differentiation to HLC in collaboration with Melissa Baxter and Sarah Withey. The western blotting, immunostaining and quantification of DE markers of figure 1 and supplementary figure 2. The assay of albumin (ALB) secretion and quantification of ALB/AAT in figure 4 (with Melissa Baxter). The assay of cytochrome P450 (CYP) 3A activity by luciferin in figure 7. Along with the associated data analysis and presentation in figures, as well as their description in results and methods chapters.
1.1 Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening

Melissa A. Baxter a, Cliff Rowe a, Jane Alder c, Sean Harrison a, Karen Piper Hanley a, B. Kevin Park b, Neil R. Kitteringham b, Chris E. Goldring b, Neil A. Hanley a

a Endocrinology & Diabetes, School of Biomedicine, Manchester Academic Health Science Centre, University of Manchester, Oxford Road, Manchester, M13 9PT, UK
b MRC Centre for Drug Safety Science, Department of Pharmacology & Therapeutics, University of Liverpool, Sherrington Buildings, Ashton Street, Liverpool L69 3GE, UK
c School of Pharmacy and Pharmaceutical Science, University of Central Lancashire, Preston PR1 2HE, UK.

1 These authors contributed equally.

Published in Stem Cell Research (2010) 5, 4-22
1.1.1 Abstract

Hepatotoxicity is an enormous and increasing problem for the pharmaceutical industry. Early detection of problems during the drug discovery pathway is advantageous to minimize costs and improve patient safety. However, current cellular models are sub-optimal. This review addresses the potential use of pluripotent stem cells in the generation of hepatic cell lineages. It begins by highlighting the scale of the problem faced by the pharmaceutical industry, the precise nature of drug-induced liver injury and where in the drug discovery pathway the need for additional cell models arises. Current research is discussed, mainly for generating hepatocyte-like cells rather than other liver cell-types. In addition, an effort is made to identify where some of the major barriers remain in translating what is currently hypothesis-driven laboratory research into meaningful platform technologies for the pharmaceutical industry. © 2010 Elsevier B.V. All rights reserved.
1.1.2 Introduction

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

1.1.2.1 The scale and importance of drug-induced liver injury

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

**Figure 1-1 Liver injury.**

The liver is the primary organ for metabolic biotransformation of xenobiotics, including drugs, and is consequently a frequent target for a variety of hepatotoxic insults. Chemically-mediated toxicity can affect any cell-type and mimic any naturally occurring disease process. Frequently, the initial toxic insult is followed by involvement of the immune system with a resulting inflammatory reaction.
Figure 1-2 The multi-tiered defence response against chemically-induced toxicity within the liver.

Diagram to show the range of defence responses adopted by the liver in reaction to increasing severity of chemical stress. Initial defence is provided through constitutive expression of antioxidant proteins and low molecular weight scavengers (e.g. glutathione), as well as phase II drug metabolizing enzymes and transporters. Subsequently these defences are bolstered through a transcriptionally induced up-regulation of defence proteins, principally orchestrated through the factor Nrf2. Finally, the cell is scheduled to undergo apoptotic self-destruction as an alternative to necrotic cell death, which carries with it the added risk of an inflammatory response.

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

1.1.2.2 The drug discovery pathway - the need for better cell models

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

Traditionally, the bottleneck in the discovery pathway was thought to be the identification of hit compounds based on, amongst other criteria, assay performance, analyses of structure-activity relationships, potency and dose response effects (Fig. 3). However, as in silico technologies for high-throughput screening have improved, attrition rates have not, indicating that the bottleneck has shifted downstream to the phase of ‘lead optimization’ (Bleicher et al. 2003). Lead optimization aims to improve the attributes of a compound as a candidate drug (or, potentially, develop a pro-drug that would only be converted to its active form after administration) by altering chemical structure, as well as considering absorption, distribution, metabolism and elimination properties. Critically, lead optimization also involves assessment of toxicity in a range of tests. Animal studies, both in vitro and in vivo, are a useful tool in the drug development process and a number of species, including rodents, dog and monkey, are commonly used for testing potential toxicity of NCEs before the first dose is administered in man. However, species differences in the activities of orthologous drug metabolizing enzymes, which may produce hepatotoxic metabolites in a species-specific manner means that extrapolation to man from such models must be undertaken with caution (Martignoni et al. 2006). Between synthetic in vitro assays of cytochrome P450 (CYP) activity and animal studies, the FDA report identified a pressing need for better in vitro cell models to broaden understanding of the biochemical and genetic basis of DILI, and to develop predictive liver toxicity biomarkers for hepatotoxicity screening; hence, the interest in human PSC research.
Figure 1-3 The drug discovery pathway.

Schematic overview of both the pre-clinical and clinical phases of drug development. ESC-derived liver cells are highly desirable as cell models during lead optimization.

The goals are two-fold and interlinked: to demonstrate the safety and effectiveness of potential lead compounds in faster time-frames, with more certainty, at lower costs; and to identify unfavourable toxicity profiles during lead optimization thereby reducing attrition rates in late-stage development.

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

1.1.3.1 Requirements for effective hepatotoxicity screening

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

Figure 1-4 The human liver and its composite cell-types.

Diagram to show the organization of different cell-types in the liver. Hepatocytes are arranged as anastomosing plates, one cell thick. Biliary epithelial cells line the bile duct and contribute to bile secretion. Bile collects in canaliculi which run along the surface of hepatocytes. Hepatic sinusoids are small blood vessels lined by discontinuous endothelial and Kupffer cells (specialized macrophages of hematopoietic origin). The sinusoids are separated from the hepatocytes by the space of Disse, which contains stellate cells. In the absence of liver injury, stellate cells at this location are considered quiescent but are thought to activate in response to inflammatory stimuli when they migrate into the liver parenchyma and deposit the excessive extracellular matrix characteristic of liver fibrosis.
1.1.3.2 Human hepatocellular carcinoma cell lines

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

1.1.3.3 Primary human hepatocyte culture

Isolated primary human hepatocytes are the current ‘gold-standard’ in vitro model as they express the entire complement of hepatocyte drug metabolizing enzymes and transporters (Hewitt et al. 2007). Dissociation and culturing purified hepatocytes in monolayer obviously compromises model complexity compared to the native organ, however, overlaying extracellular matrix enhances maintenance of the differentiated hepatocyte phenotype, including cell polarity, for days or weeks (Moghe et al. 1996, LeCluyse 2001). Co-culture of hepatocytes with epithelial cells (Rogiers and Vercruysse 1993), stellate cells (Riccalton-Banks et al. 2003) and pancreatic cells (Kaufmann et al. 1999) has also been investigated as a step towards designing systems with enhanced liver complexity. Despite this, there are many issues that remain problematic with the use of primary human hepatocytes. Supply is scarce as relatively small numbers can be isolated from tissue derived from planned surgical resections. Large numbers of hepatocytes may occasionally become available from whole organs deemed unsuitable for transplantation, but by nature this tissue is often of poorer quality and unpredictable in supply. Such issues have stimulated research on cryopreservation (Terry and Hughes 2009), which has made progress, although there is inevitable variation between batches (Roymans et al. 2004). As
a toxicity screening platform for the pharmaceutical industry, batch-to-batch consistency and ability to ship cryopreserved cells without loss of function are important. Even in optimised culture systems, hepatocytes still lose the differentiated phenotype that is essential for meaningful study of drug metabolism and toxicity (Elaut et al. 2006). Dedifferentiation includes losing the ability of certain CYPs and phase II genes to be induced by reference compounds (Park et al. 1996), in part due to the activation of stress signalling pathways and the generation of nitric oxide (Lopez-Garcia 1998, Rodriguez-Ariza and Paine 1999, Vernia et al. 2001, Sidhu et al. 2004, Olsavsky et al. 2007, Page et al. 2007, Aitken et al. 2008). Nitrite levels are inversely correlated to CYP levels and activity; the latter decline with increased nitrosylation, haem loss and degradation (Lopez-Garcia 1998, Vernia et al. 2001, Aitken et al. 2008). Nitric oxide-producing stimuli also reduce the levels of liver enriched nuclear factors and nuclear receptors (Beigneux et al. 2002, Fang et al. 2004).

Differential pharmacodynamic and toxic responses in vivo and in vitro arise from the great diversity of metabolic phenotypes in human populations. These may be genetic, environmental, dietary, occupational, disease-related, or drug-induced and explain, in large part, the differences demonstrated by pools of hepatocytes isolated from different donors (Ponsoda et al. 2001). This genotype-dependent expression of important CYPs, such as CYP3A isoforms, CYP1A2, CYP2A6, CYP2B6, CYP2D6, and several CYP2C isoforms, would cause highly variable toxicity readouts for NCEs. Ideally, this genotypic diversity needs to be reflected in banks of primary hepatocytes or equivalent depositories of PSC-derived cells (Ingelman-Sundberg et al. 2007). Not all variability in gene expression is due to sequence differences; the expression of microRNAs (miRNA) and gene methylation are additional control mechanisms that may play a role in the severe down-regulation of CYP expression in isolated and primary cultured cells, and cell lines (Ingelman-Sundberg et al. 2007, Gomez and Ingelman-Sundberg 2009). Methylation of CpG islands in the gene promoters of several of the most important CYPs have been identified, the transcripts from which may also be regulated by miRNAs interacting with the 3’UTR, as is the case for CYP3A4 and CYP2B6 (Ingelman-Sundberg et al. 2007).

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

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

1.1.4.1 Development of the liver

In vitro protocols for differentiating PSCs to hepatocytes draw heavily on knowledge of how the liver normally develops and matures during embryogenesis and the fetal period (Fig. 5). The three germ layers of the embryo are formed during gastrulation which begins at E6.5 in mice (Kubo et al. 2004). During this period, epiblast cells fated to become definitive endoderm undergo epithelial-mesenchymal transition (EMT) at the anterior primitive streak under the influence of WNT and high levels of Nodal signalling (Kubo et al. 2004, Murry and Keller 2008, Nakanishi et al. 2009). Folding turns the flat sheet of definitive endoderm, regulated by the transcription factors SRY box 17 (SOX17), GATA4 and forkhead box A2 (FOXA2), into a gut tube (Wells and Melton 1999). Hepatogenesis
begins at E8.5-9 from the ventral foregut endoderm in progenitor cells that harbour an equal capacity to form the ventral bud of the pancreas (Kamiya et al. 1999, Deutsch et al. 2001). Hepatic specification of these bipotent cells is induced by dual signalling from two adjacent mesodermal sources: fibroblast growth factor (FGF) 1, FGF2 and FGF8 from the neighbouring cardiac mesoderm; and bone morphogenic protein (BMP) 2 and BMP4 from the septum transversum (Jung et al. 1999, Rossi et al. 2001, Chung et al. 2008). Once specified, the hepatoblasts proliferate under autocrine signals and ones from surrounding angioblasts, and invade the stroma of the septum transversum (Jung et al. 1999, Matsumoto et al. 2001, Lemaigre and Zaret 2004). They are bipotent, expressing markers for fetal hepatocytes [e.g. hepatocyte nuclear factor (HNF) 4α, HNF6, alpha-fetoprotein (AFP) and albumin (ALB), and biliary epithelium (e.g. cytokeratin (KRT) 17 and KRT19)] (Tanimizu and Miyajima 2004, Lavon and Benvenisty 2005) (Fig. 5). At E10, haematopoietic cells colonize the nascent liver bud. This induces Notch signalling, which acts to differentiate the bipotential hepatoblasts by promoting or inhibiting differentiation into either biliary duct epithelium or hepatocytes respectively (Tanimizu and Miyajima 2004, Lavon and Benvenisty 2005). The haematopoietic cells also produce the cytokine, oncostatin M (OSM), which, in combination with glucocorticoid, acts as a liver maturation factor (Kinoshita et al. 1999). Hepatocyte growth factor (HGF) from the septum transversum and surrounding nonparenchymal liver cells also plays important roles over a long time course by promoting hepatocyte proliferation, survival and maturation (Zaret 2000, Kamiya et al. 2001). Complete hepatocyte maturation only occurs after birth (Kamiya et al. 2001).
Liver development in vivo and hepatocyte differentiation in vitro are depicted with examples of gene expression associated with each developmental or differentiation stage. Exogenous factors are shown that are typically added in vitro to induce differentiation.

### 1.1.4.2 Differentiating pluripotent stem cells towards hepatocyte-like cells

At present, efforts to generate hepatocyte-like cells have been predominantly from ESCs rather than iPSCs, although the first advances with the latter cell-types have recently been published (Song et al. 2009, Sullivan et al. 2009). So far, methods developed using ESCs have also been found broadly applicable using iPSCs (Gareth et al., Song et al. 2009). Various strategies have been applied, most of which aim to simulate in vivo liver development in the in vitro setting (Fig. 5). These protocols are reviewed along with the corresponding characterisation studies in Table 2.

### Table 1 Characteristics for defining human PSC-derived hepatocyte-like cells.

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

**B**

<table>
<thead>
<tr>
<th>Functional assessment</th>
<th>Method overview</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen storage</td>
<td>Periodic Acid-Schiff staining</td>
<td>Useful but also occurs in muscle and undifferentiated ESCs</td>
<td>(Gomori 1952)</td>
</tr>
<tr>
<td>Urea production</td>
<td>Measurement of synthesis and excretion by detection in cell-free conditioned medium</td>
<td>Specific hepatocyte function</td>
<td>(Pless et al. 2006)</td>
</tr>
<tr>
<td>Albumin secretion</td>
<td>Measurement of protein in cell-free conditioned medium</td>
<td>Relatively insensitive test - other functional properties are lost before albumin secretion</td>
<td>(Dich et al. 1987)</td>
</tr>
<tr>
<td>Low density lipoprotein (LDL) uptake and metabolism</td>
<td>Detection of labelled intracellular uptake and degradation</td>
<td>Useful but also occurs in vascular endothelial cells and macrophages</td>
<td>(Voyta et al. 1984)</td>
</tr>
<tr>
<td>Indocyanine green (ICG)</td>
<td>Hepatocytes in culture take up and secrete ICG dye over 6 h</td>
<td>Difficult to apply to cultured cells due to lack of functional bile excretion system in vitro</td>
<td>(Pless et al. 2006) (Yamada et al. 2002)</td>
</tr>
</tbody>
</table>

**C**

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

A. Genes expressed in human fetal hepatoblast, mature hepatocyte and biliary epithelial cells to discriminate these cell-types from others that might arise during PSC differentiation. ‘% ESTs’ refers to the percentage of expressed sequence tags derived from liver in the Unigene database; high values indicate expression that is restricted to liver. No gene is uniquely expressed in liver, however, the expression of ‘discriminatory genes’ should not be detected in fetal hepatoblast, mature hepatocyte or biliary epithelial cell respectively. B. Functional analyses that can be undertaken to assess human PSC-derived hepatocyte-like cells in comparison to freshly isolated human primary hepatocytes. C. CYP enzymes expressed in mature hepatocytes along with their metabolic substrates and reference compounds known to induce their expression and/or activity

<table>
<thead>
<tr>
<th>Table 1-1 Characteristics for defining human PSC-derived hepatocyte-like cells</th>
</tr>
</thead>
</table>

1.1.4.2.1 In vitro simulation of in vivo liver development

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

**Soluble growth factors.**

The first protocol to describe differentiation of ESCs towards hepatocytes used embryoid body (EB) formation to induce initial differentiation (Hamazaki et al. 2001). EB formation by ESC aggregation, potentially with subsequent plating onto adhesive substrates (such as Matrigel or collagen) as EB outgrowth cultures (Itskovitz-Eldor et al. 2000), serves as an in vitro mimic of gastrulation and subsequent lineage specification. However, the differentiation is random (Kubo et al. 2004, Soto-Gutierrez et al. 2006, Momose et al. 2009, Pei et al. 2009). To prioritise hepatocyte differentiation, cultures have been treated with exogenous factors and the resulting hepatocyte-like cells characterised by their gene expression profile and characteristics (Duan et al. 2007, Momose et al. 2009)(Table 2). Subsequent protocols have aimed to avoid the inherent random differentiation in EB-based protocols by the direct induction of ESCs cultured in monolayer. As a mimic for the in vivo Nodal and WNT signalling at the anterior end of the primitive streak, initial definitive endoderm differentiation of ESCs has been routinely induced by WNT3a and high concentrations of Activin A (commonly 100 ng/ml) (Cai et al. 2007, Nakanishi et al. 2009,
Zhao et al. 2009). Conversely, phosphatidylinositol 3-kinase (PI3K) is known to block endoderm differentiation from ESCs (D'Amour et al. 2005), such that low serum concentrations or specific PI3K inhibitors (e.g. LY294002) have been used alongside Activin A (McLean et al. 2007) (Nobuaki Shiraki 2008).

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

For final maturation of hepatocyte progenitors, specialised hepatocyte growth medium has been devised (Jasmund et al. 2007) with a number of added maturation factors. These factors typically include HGF, shown to support the expansion of fetal hepatocytes (Kamiya et al. 2001), OSM, known to induce differentiation and maturation of fetal hepatocytes (Kinoshita et al. 1999, Kamiya et al. 2001) and dexamethasone (DEX), a potent synthetic glucocorticoid, which induces the activity of hepatocyte enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT) and members of the CYP family (Duanmu et al. 2002, Neta Lavon 2005).

**Co-culture with supporting cell-types.**

Similar to the recognised benefits of culturing PSCs on inactivated mouse embryonic feeder cells (MEFs) (Thomson et al. 1998), differentiating cells in vitro toward hepatocytes can benefit from contact with other cells (Fair et al. 2003). During development hepatoblasts interact with both the cardiac and septum transversum mesoderm; in teratomas, hepatocyte-like cells have been observed to differentiate alongside cardiac cells (Lavon et al. 2004). ESCs co-cultured with both embryonic chick mesoderm (Fair et al. 2003) and a mesoderm-derived cell line (M15) (Nobuaki Shiraki 2008) demonstrated enhanced hepatocyte differentiation. Other examples include the use of human non-parenchymal liver cell lines, which have been shown to support mature hepatocyte culture by the production of hepatocyte mitogens (Kang et al. 2004). Similarly, Zhao and colleagues found that ESC-derived hepatic progenitor cells could be expanded upon murine embryonic stromal cells (Zhao et al. 2009). Alternatively, feeder cell lines have been genetically modified to produce specific growth factors, such as FGF2, in order to enhance hepatocyte differentiation (Pei et al. 2009).
Table 2  Summary of studies reporting the differentiation of human PSCs to hepatocyte-like cells

<table>
<thead>
<tr>
<th>Ref</th>
<th>Differentiation method</th>
<th>Characterisation</th>
<th>Liver-associated markers</th>
<th>Functional assessment</th>
<th>Comments/limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monolayer Co-culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ECM</td>
<td>Factors added</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT-PCR</td>
<td>ICC</td>
<td>WB</td>
<td>FACS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFP</td>
<td>AAT</td>
<td>HNF4</td>
<td>TAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Albumin secretion</td>
<td>Glycogen storage</td>
<td>ICG uptake &amp; excretion</td>
<td>LDL uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CYP activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>In vivo transplant</td>
</tr>
</tbody>
</table>

(Rambhatla et al. 2003)  
+ + M  
EB + + DMSO, Na Butyrate, HGF  
Monolayer Co-culture  
ECM  
Factors added  
RT-PCR + + - -  
ICC  
WB  
FACS  
AFP + + + -  
AAT -  
HNF4 +  
TAT -  
Albumin secretion -  
Glycogen storage +  
ICG uptake & excretion -  
LDL uptake -  
CYP activity -  
In vivo transplant -  
Comments/limitations  
AFP not detected in Na butyrate-treated cultures.

(Lavon et al. 2004)  
+ -  
EB + - FGF1, FGF2, BMP4, HGF  
Monolayer Co-culture  
ECM  
Factors added  
RT-PCR + + - +  
ICC  
WB  
FACS  
AFP + - - -  
AAT +  
HNF4 -  
TAT -  
Albumin secretion -  
Glycogen storage -  
ICG uptake & excretion -  
LDL uptake -  
CYP activity -  
In vivo transplant -  
Comments/limitations  
Each growth factor added individually, not altogether. Very simple differentiation protocol.

(Cai et al. 2007)  
+ + MEF  
EB + + AA, FGF4, BMP2, HGF, OSM, DEX  
Monolayer Co-culture  
ECM  
Factors added  
RT-PCR + + - -  
ICC  
WB  
FACS  
AFP + + - -  
AAT +  
HNF4 +  
TAT +  
Albumin secretion +  
Glycogen storage +  
ICG uptake & excretion +  
LDL uptake +  
CYP activity +  
In vivo transplant +  
Comments/limitations  
CYP activity levels not compared to adult hepatocyte control. Very low levels of liver integration in vivo.

(Duan et al. 2007)  
+ + C  
EB + + DEX  
Monolayer Co-culture  
ECM  
Factors added  
RT-PCR + + - +  
ICC  
WB  
FACS  
AFP + + + +  
AAT +  
HNF4 +  
TAT +  
Albumin secretion +  
Glycogen storage +  
ICG uptake & excretion +  
LDL uptake +  
CYP activity +  
In vivo transplant +  
Comments/limitations  
Method relies on spontaneous hepatocyte ESC differentiation and microdissection for hepatocyte enrichment. Variable TAT expression.
<table>
<thead>
<tr>
<th>Study</th>
<th>Culture Medium</th>
<th>Source Cells</th>
<th>Growth Factors</th>
<th>Differentiation</th>
<th>CYP Enzyme Activity</th>
<th>Hepatocyte Maturation</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ek et al. 2007)</td>
<td>-</td>
<td>MEF</td>
<td>FGF2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Hay et al. 2007)</td>
<td>-</td>
<td>M</td>
<td>DMSO, HGF, OSM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(Agarwal et al. 2008)</td>
<td>-</td>
<td>C</td>
<td>AA, FGF4, HGF, OSM</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Baharvand et al. 2008)</td>
<td>-</td>
<td>M</td>
<td>FGF2, Noggin, aFGF, FGF4, HGF, DEX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(Chiao et al. 2008)</td>
<td>+</td>
<td>FGF1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

No CYP activity despite detection of CYP transcript and protein.

Low efficiency: only foci of hepatocyte-like cells, only 10% of which were positive for ICG. No positive control for CYP activity assay.

No data on CYP enzyme activity levels. Low/sporadic liver integration and differentiation.

No expression of G6P, marker of hepatocyte maturation. Adult primary hepatocytes not used as control in functional assays.

AFP positive cells selected by reporter gene expression. Very simple differentiation protocol.
| Study (Author et al. Year) | AA, Na Butyrate, DMSO, HGF, OSM | M15 cells | AA, HGF, FGF2, LY294002, HGF, DEX | MEF | Fet lap liver cell | Low CYP protein+ activity levels compared to adult hepatocyte control. | Aim of study to assess the effect of extracellular matrices on hepatic differentiation. No functional tests. | Low efficiency: 9% albumin positive cells after 40 days differentiation. | Transplantation of ASGR sorted population enriched for liver markers. Recipients developed adenocarcinoma. | CYP activity levels only compared to undifferentiated ESC no positive adult primary hepatocyte control. | No data on CYP activity levels. Limited panel of |
line markers used to characterize hepatocytes.

| (Song et al. 2009) | - | - | MEF | AA, HGF, BMP2, FGF4, KGF, OSM, DEX | + | + | - | - | + | + | + | - | - | + | - | - | - | - | Very low levels of albumin secretion, urea synthesis, albumin secretion and CYP3A4 activity in comparison to adult hepatocytes. |
| (Zhao et al. 2009) | - | + | STO | AA, BMP2, FGF4, HGF, OSM, DEX | + | + | - | + | + | + | + | + | + | + | + | + | + | - | Albumin secretion and CYP activity levels not compared to adult hepatocyte control. |

Table 1-2 Summary of studies reporting the differentiation of human PSCs to hepatocyte-like cells
Extracellular matrix.

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

1.1.4.2.2 Epigenetic manipulation of hepatocyte-like differentiation from embryonic stem cells

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

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

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

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

A summary of characterisation from different studies is provided in Table 2. The initial formation of definitive endoderm has been characterised by the transient expression of the primitive streak marker Brachyury, and a combination of FOXA2, GATA4 and SOX17; (D'Amour et al. 2005)(Fig. 5). The subsequent generation of hepatoblasts has been defined by ALB and AFP expression (Soto-Gutierrez et al. 2007). More mature hepatocyte-like cells, suggested by characteristic epithelial morphology, have been described according to immunostaining for a combination of ALB along with CK18, and transcription factors, such as HNF4α, CCAAT/enhancer-binding protein α (CEBPα) and CEBPβ, in the absence of AFP (Monaghan et al. 1993, Duan et al. 2007, Baharvand et al. 2008). Similar status has been inferred by expression of a range of liver-specific enzymes such as CYPs, TAT and AAT (Cai et al. 2007, Duan et al. 2007, Zhao et al. 2009). In some studies, functional characteristics have been obtained. During repair following liver injury, transplanted ESC-derived hepatocyte-like cells carried a selective advantage (Shafritz and Dabeva 2002), and have been shown to integrate and differentiate into hepatocytes in host liver (Gouon-Evans et al. 2006, Agarwal et al. 2008), secrete ALB and AAT (Duan et al. 2007, Basma et al. 2009), improve hepatic function and increase survival rates (Soto-Gutierrez et al. 2006, Ishii et al. 2007). Similarly, their use in bioartificial liver devices improved short-term (5-day) survival rates of rats with fulminant hepatic failure (Cho et al. 2008).
1.1.5 Unmet demands and future challenges for hepatocyte-like cells generated from pluripotent stem cells

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

1.1.5.1 Standardizing initial characterisation of hepatocyte-like cells

At present the characterisation of hepatocyte-like cells generated from human PSCs has tended to reflect the hypothesis-driven experimentation of individual, independent research laboratories. Most protocols have described the appearance of cells with typical hepatocyte morphology, however, there has been a general lack of agreed standards, equivalent for instance to the ‘minimum information about a microarray experiment (MIAME)’ parameters required to deposit expression microarray data in public repositories (Brazma et al. 2001, Brazma 2009). In redress, Sancho-Bru et al. have made very helpful recommendations for the characterisation of stem cell derived hepatocyte-like cells (Sancho-Bru et al. 2009), highlighting minimal criteria by which different protocols can be compared. In Table 1A, we also provide a profile of gene expression, reliant on both positive and negative markers, attempting to discriminate hepatocyte-like and biliary-like cells from other cell-types. The major problem is that genes expressed during hepatocyte differentiation are also commonly expressed in other organs and tissues (e.g. AFP in extra-embryonic endoderm (Gualdi et al. 1996), and FOXA2 in other endodermal and ectodermal tissues (Neta Lavon 2005)). Analysing the origin of human expressed sequence tags (ESTs) in the Unigene database reinforces the scale of this problem: only 21% of ESTs for human AFP originated from liver; most came from human fetal heart libraries making the inclusion of ‘negative control’ genes, such as MLC2V, particularly important (Table 1A). This issue can compromise differentiation strategies reliant on reporter genes expressed under the control of regulatory elements from a single ‘hepatocyte-specific’ gene (Soto-Gutierrez et al. 2006, Duan et al. 2007, Ishii et al. 2007). Where AFP-positive cells have been selected via reporter gene expression, other hepatocyte markers including TAT and tryptophan 2,3-dioxygenase (TDO2) have been weakly detected and albumin has only been expressed in a minority of cells, suggestive of significant extra-embryonic differentiation (Ishii et al. 2008).
When it comes to mature hepatocytes, commonly used markers can also be expressed in other tissues, for example HNF4α in the kidney, pancreas, testis and intestine (Drewes et al. 1996). However, highly restricted gene expression profiles are available for mature hepatocytes. Although CYPs are surprisingly widely expressed, all bar one human EST for the organic anion transporter OATP2 (officially known as SLCO1B1) and AAT (officially known as SERPINA10), arose from liver (the others were from breast and fetal heart, respectively). When coupled to the expression of other markers, like TDO2 and TAT, it is possible to clearly define the human hepatocyte by the expression of surprisingly few genes. The same does not appear true of the biliary epithelial cell, where greater reliance on the absence of negative markers becomes important (Table 1A).

1.1.5.2 Functional attributes of hepatocyte-like cells for drug toxicity platforms

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

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

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

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

The opposite perspective from trying to isolate pure hepatocyte-like cells is to actively encourage protocols that incorporate other liver cell-types thereby trying to create a more complete in vitro mimic of agents capable of inducing liver fibrosis.

1.1.5.4 Phenotypic stability and scale-up of hepatocyte-like cells derived from pluripotent stem cells

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

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

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

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

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

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

1.1.5.7 Other commercial considerations and practicalities

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

1.1.6 Summary

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

1.1.7 Acknowledgments

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

As well as the endoderm derived hepatocytes of the parenchyma the liver contains mesoderm derived cells. A full description of the development of the liver cannot omit the contribution of these cells to the development of the organ. Knowledge of the development of all the cells of the liver as well as of their formation into functional structures is necessary for understanding the potential interactions between the differentiating cells, which can then be used for protocol development and accurate characterisation of PSC derived hepatic cells.

1.2.1 Septum transversum contributes to liver development

Development of the hepatic parenchyma requires interactions from the surrounding mesoderm from the earliest stages. Mesodermal cell types are later incorporated into the liver during development giving rise to the stromal cells of the adult liver. These non-parenchymal cells of the liver play crucial roles in the development of hepatocytes and cholangiocytes, and in the functions of the adult liver. The area of the developing foregut which gives rise to the hepatic parenchyma is situated next to the lateral plate mesoderm, part of which will develop into the septum transversum. The septum transversum mesenchyme (STM) in turn gives rise to the mesodermal cell types of the developed liver, such as; the mesothelial cells (MCs), fibroblasts and liver sinusoidal endothelial cells (LSECs). The aorta-gonad-mesonephros (AGM) like the septum transversum is also derived from the lateral plate mesoderm. It is an early site of haematopoiesis, generating hematopoietic cells which migrate to the liver as it in turn becomes a site of fetal haematopoiesis. This colonisation of the fetal liver with hematopoietic cells happens shortly after the hepatic progenitors invade the STM (Si-Tayeb et al. 2010).

The STM surrounding the liver bud is positive for GATA4, a zinc finger transcription factor (Si-Tayeb et al. 2010). GATA4 knockout (KO) prevents the formation of the STM in the embryo (Watt et al. 2004) and also the expansion of the liver bud (Watt et al. 2007), showing that the STM is required for early hepatic development, around embryonic day (E) 8.0 in mice. At E8.5 both BMP4 and GATA4 are highly expressed in the STM (Rossi et al. 2001) with GATA4 having been shown to regulate BMP4 expression (Nemer and Nemer 2003). BMP4 KOs delay hepatic expansion and BMP inhibition prevents hepatic specification in vitro (Rossi et al. 2001). However the STM is not the only source of BMP4 and other BMP proteins (BMP2) are present (Si-Tayeb et al. 2010). The difference
seen between the BMP4 KO and BMP inhibition along with the presence of other BMP proteins suggests there is some redundancy in the BMP signalling during liver development.

The cardiac epicardium, along with the STM, is also derived from lateral plate mesoderm and found adjacent to the developing hepatic endoderm (Si-Tayeb et al. 2010). This cardiac mesoderm also plays a role in inducing hepatic specification in the ventral endoderm at E8.5 (Gualdi et al. 1996).

At E9.5 the STM is invaded by the delaminating ventral foregut endoderm giving rise to the hepatoblasts (Medlock and Haar 1983, Bort et al. 2006, Si-Tayeb et al. 2010). This is the event which mixes the endoderm and mesoderm lineage cells of the liver with the STM trapped between the growing cords of hepatoblasts and its endothelial cells (ECs), as revealed by electron microscopy (Enzan, Himeno et al. 1997). The delamination of the hepatoblasts is made possible by matrix metalloproteinases (MMP), such as MMP2, which degrade extra cellular matrix (ECM) and are found in the surrounding mesenchyme (Margagliotti et al. 2008). The migrating hepatoblasts which invade the STM are at that point bipotent and have not yet differentiated down the hepatocyte or cholangiocyte lineages (Si-Tayeb et al. 2010).

The cells of the trapped STM will become the stromal cells of the adult liver; the sinusoids, MCs and hepatic stellate cells (HSC). These stromal cells have been shown to interact with other hepatic cell types and play important roles during development. At E9.5 the ECs of the mouse promote the growth of the hepatic parenchymal cells (Matsumoto et al. 2001). KO of HLX in mice, a gene expressed by STM but not endoderm derivatives, caused hypoplasia of the liver at E12.5, the hepatic progenitors successfully formed chords by E9.5 but then failed to proliferate (Hentsch et al. 1996). KO of LIM/homeobox protein 2 (LHX2), expressed by both STM and HSCs, caused fibrosis and increased deposition of ECM in mouse livers and also increased hepatocyte gene expression and disrupted sinusoidal architecture (Kolterud et al. 2004). HSCs express HGF (Burt 1999) as do isolated activated leukocyte cell adhesion molecule (ALCAM) high cells from the fetal liver, which represent the MCs and subMCs (Asahina et al. 2009). HGF via its interaction with the B-catenin pathway promotes hepatocyte proliferation (Apte et al. 2006), as does pleiotrophin also secreted by the ALCAM high cells (Asahina et al. 2009) highlighting there potential for involvement in parenchymal development. Human liver fibroblasts have been shown to support the co-culture of hepatocytes in vitro (Jodon de Villeroche and Brouty-Boye 2008).
During human development hepatic specification of the foregut endoderm has occurred by 4 weeks post conception (wpc). At 5wpc it infiltrates the STM with the trapped mesenchyme thought to become HSCs, based on their similar morphology (Enzan et al. 1983, Enzan et al. 1997). The STM as the source of HSCs in mouse liver development is supported by the shared ability to store vitamin A in lipids (adult HSCs) and shared expression of the transcription markers LHX2 (Kolterud et al. 2004) and FOXF1 (Lamers et al. 1987, Kalinichenko et al. 2002). LHX2 is co-expressed with DES in adult mouse HSCs (Kolterud et al. 2004) and maintains their quiescent phenotype (Gaasbeek Janzen et al. 1988). More specifically a mesothelial origin has also been demonstrated for HSCs in human (Loo and Wu 2008) and mouse (Asahina et al. 2009).

In humans the vasculature of the STM gives rise to the sinusoids of the liver, which then further develop by angiogenesis (Enzan et al. 1983, Couvelard et al. 1996, Collardeau-Frachon and Scoazec 2008). While lineage tracing in avian embryos has shown that the STM along with the proepicardium give rise to mesothelial cells which in turn generate ECs and HSCs, forming the sinusoids (Perez-Pomares et al. 2004).

The mesodermal origin of MCs, subMCs, HSCs and perivascular mesenchymal cells (PMCs) in E13.5 mouse livers has been demonstrated using cell lineage analysis to trace mesoderm posterior 1 (MESP1) derived cells (Asahina et al. 2009). MESP1 is expressed during gastrulation in the mesoderm and MESP1 cells contribute to the lateral plate mesoderm at E5-7 (Saga et al. 1999). MESP1 lineage tracing in E9-9.5 embryos revealed that the STM is derived from these cells, prior to the development of the liver and the STM in turn gives rise to mesothelial and mesenchymal cells in the liver. MCs, subMCs, HSC and PMCs were all found to be derived from the MESP1 cells while parietal mesothelium and Kupffer cells were not. Also MESP1 derived were the endothelial cells of large vessels but not the LSECs, CD45+ cells, or foregut endoderm (Asahina et al. 2009, Asahina et al. 2011).

### 1.2.2 Mesenchymal, mesothelial and hepatic stellate cells

The liver mesenchyme, mesothelium and perisinusoidal cells share a common developmental origin, the STM. The liver mesenchyme further differentiates to form both transient and adult liver cell types; the MCs, subMCs, HSCs, PMCs and myofibroblasts.

MCs are an epithelial cell type derived from mesenchymal cells. The MCs of the liver form a single cell layer around its external surface surrounding the ECM known as Glisson’s capsule and expressing the epithelial associated cytokeratins.
SubMCs are mesenchymal cells which are found directly beneath the mesothelium. Several groups have also reported mesenchymal populations consistent with the developmental cell population of subMCs such as capsular fibroblasts beneath the MCs in rat (Bhunchet and Wake 1992) and neural cell adhesion molecule positive (NCAM) subMCs in human (Loo and Wu 2008). A sub-mesothelial layer of transitional cells is also found during avian liver development (Perez-Pomares et al. 2004).

PMCs are mesenchymal cells which surround the developing veins and can later be distinguished as separate populations in the developed liver including portal fibroblasts (PFB), smooth muscle cells (SMC), and fibroblasts.

HSCs are found between the LSECs and the hepatocytes in the space of Disse (Friedman 2008). HSCs of the adult liver express mesenchymal cell markers, store vitamin A and have long dendritic processes which extend along the sinusoids (Asahina et al. 2009). Upon liver damage HSCs lose their stored vitamin A, become activated and differentiate into myofibroblasts expressing α-smooth muscle actin (αSMA). Fetal Liver HSCs, however, lack the vitamin A storage commonly used to characterise and isolate adult HSCs (Kubota et al. 2007).

### 1.2.2.1 Animal models

Asahina et al defined three populations of mesenchymal cells within the developing mouse liver; HSCs found in the parenchyma which are DES+/p75NTR+/αSMA+/-, subMCs located beneath the mesothelium which are DES+/p75NTR+/ALCAM+/PDGFRα+, and PMCs found near the veins which are DES+/p75NTR+/αSMA+ (Asahina et al. 2009). MCs were also found to be ALCAM+ and in contact with a COLIV matrix. At E12.5 the MCs are panCK+/DES-ve and by E15.5 they have become DES+ (Asahina et al. 2009, Asahina et al. 2011). SubMCs were separated from MCs by a COLIV containing ECM layer, seen from E11.5 (Asahina et al. 2011).

It was further shown that the subMCs can detach from the basal lamina and migrate into the parenchyma and hypothesised that these migratory subMCs transition to HSCs. In support of this it was found that ALCAM+ cells isolated from fetal liver acquire characteristics of HSCs when cultured. Such as lipid storage when cultured on collagen and differentiation to an αSMA+ fibroblast morphology when grown on plastic (Asahina et al. 2009). p75 neurotrophin receptor (p75NTR) positive cells, representing the HSCs, PMCs and subMCs, isolated from E11.5 livers become αSMA+ and glial fibrillary acidic protein (GFAP) positive in vitro (Suzuki et al. 2008). While p75NTR+ subMCs expressed αSMA but not GFAP when cultured in vitro (Asahina et al. 2009).
Asahina theorise that the combination of proliferation and migration seen in the subMCs and MCs and their ability to form αSMA+ myofibroblasts in vitro means they maybe the precursors of HSCs (Asahina et al. 2009).

Asahina et al then used Wilms tumor 1 (WT1) lineage tracing to follow the STM cells as they develop into MC/subMCs, HSCs and PMCs. At E9-E9.5 the STM adjacent to the foregut endoderm expresses WT1, ALCAM and DES while WT1 is absent from the ECs and the endoderm. They demonstrated that the WT1+ STM cells gave rise to WT1+ MC and subMC at the liver surface which then migrate inwards and generate the WT1-ve HSCs and PMC during morphogenesis. Examination of the non-mesenchymal cell types in the WT1 lineage tracing model found no contribution of these cells to LSECs, Kupffer cells or hepatoblasts (Asahina et al. 2011).

Quantification of this lineage tracing showed increasing numbers of lineage+ cells within the liver from E10.5 to E13.5. While normalization of the quantification data to the labelling efficiency of the starting population showed that the MC/subMC derived HSCs and PMCs are proliferative. Cells labelled (WT1+) at E10.5 gave rise to ~24% of HSCs and PMCs in the fetal mouse liver (Asahina et al. 2011) which leaves the potential for other sources of HSCs.

By later stages of development the PMC cells have begun to differentiate into distinct populations. At E18.5 fibroblasts found in the pericentral mesenchyme are DES+/lineage+/JAG1-ve/αSMA-ve. In the periportal mesenchyme two lineage+ populations were found, SMCs which were αSMA+ and PFBs which are jagged1 positive (JAG1) (Asahina et al. 2011).

### 1.2.2.2 Human data

Desmin (DES) is an intermediate filament found in muscle and mesenchymal cells. DES marks both pericytes in general and the liver specific pericytes HSCs during development (Lee et al. 2007) but not the adult quiescent HSCs (qHSC) (Schmitt-Graff et al. 1991). In contrast to the ubiquitous expression of DES in mouse HSCs during disease it is only expressed by a small number of activated HSC (aHSC) in human adult liver (Geerts 2001, Zhao and Burt 2007). αSMA is also expressed in HSCs during development and in adult liver aHSCs (Schmitt-Graff et al. 1991, Geerts 2001).

Despite these differences in marker expression of the mesenchymal cell types during development in mouse and humans, the model from Asahina et al’s mouse lineage tracing studies is supported by developmental data from human embryos. Loo et al found similar
marker expression between the STM and the subMCs and hypothesised that the subMCs may be derived from them (Loo and Wu 2008). Human subMCs were described as pockets of cuboidal cells beneath the mesothelium of the liver in 7-10wpc embryos, these were rare at 11wpc and absent at later stages. They were NCAM+/DES+/αSMA-ve/CD34-ve/ECAD-ve (Loo and Wu 2008). The cuboidal shaped cells showed a transition to spindle shaped ones and formed columns penetrating into the parenchyma, often associated with portal tracts. The edges of these mesothelial columns show cells with HSC morphology with extended processes. Cluster of differentiation 34 (CD34) positive cells were located in the sinusoids at this early stage revealing the presence of ECs (Loo and Wu 2008).

Human MCs expressed NCAM up to 8wpc but not after and were always αSMA-ve, whereas DES was still expressed until at least 20wpc (Loo and Wu 2008). The STM and subMCs were always epithelial cadherin (ECAD) negative while MCs become ECAD+ at 18-20wpc confirming their epithelial characteristics (Loo and Wu 2008).

NCAM is also a marker for human adult HSCs but not portal fibroblasts, central vein myofibroblasts or vascular SMCs (Knittel et al. 1996). NCAM staining was found to be positive in fetal HSCs revealing their localisation within the sinusoids of the human liver from 7wpc; it was concentrated in the processes of the cells. Staining showed NCAM+ expression in the subMC layer prior to staining in the sinusoids (Loo and Wu 2008). Loo et al.’s (Loo and Wu 2008) description of fetal HSCs from 7wpc matches that of other groups (Enzan et al. 1983, Enzan et al. 1997, Kolterud et al. 2004).

At 7-8wpc the subMC area showed the highest concentration of NCAM+ cells, these cells became less frequent as development progressed. At 18-20wpc NCAM+ cells were located in the space of Disse, they were most frequently located beneath the mesothelium and next to the portal tracts. Desmin staining also followed this same pattern (Loo and Wu 2008). This fits with the mouse model that the HSCs are derived from the mesothelial cells and migrate in (Asahina et al. 2009, Asahina et al. 2011). As they would be expected to be more frequently located near their MC source at early developmental stages until they reached homogeneity throughout the parenchyma as the liver matures.

From 15wpc onwards desmin staining was stronger when compared with NCAM, which itself decreases after 15wpc, and the HSCs became αSMA+ which highlighted a greater number of these cells than NCAM or DES (Loo and Wu 2008) possibly indicative of separate populations. NCAM-ve HSCs have previously been reported in 16wpc and older fetal livers (Lee et al. 2007) which agrees with the loss of NCAM seen by Loo et al.
αSMA expression has been found in developing HSCs at different time points by different groups; from 15wpc (Loo and Wu 2008), in the embryo (Schmitt-Graff et al. 1991), from 25wpc (Cassiman et al. 2002) and in the third trimester (Enzan et al. 1997).

1.2.3 Hepatic stellate cells activation in fibrosis, and development?

Liver injury causes the release of cytokines and reactive oxygen species from damaged cells which can lead to the activation of the HSCs and their differentiation to myofibroblasts (Bataller and Brenner 2005, Popov and Schuppan 2009). This induces their production of inflammatory cytokines and excessive ECM deposition. Liver fibrosis is an accumulation of ECM, specifically COL1, caused by chronic injury (Bataller and Brenner 2005). This ECM is produced by αSMA+ myofibroblasts. Fibrosis can go into regression if the source of the injury is removed, this is linked with reductions; in ECM and cytokine production, and number of myofibroblasts (Iredale et al. 1998, Bataller and Brenner 2005, Kisseleva et al. 2012).

There are several mesenchymal cell subpopulations in the liver that share morphology and have overlapping marker expression (Cassiman et al. 2002). These include; HSCs found in the space of Disse, portal fibroblasts found in the mesenchyme, periductular fibroblasts of the portal tract (Tuchweber et al. 1996), vascular SMC in the walls of veins and arteries, myofibroblasts found around the central vein (second layer cells) and fibroblasts in Glisson’s capsule (Bhunchet and Wake 1992).

Injury induced fibrogenesis is not limited to HSCs, other possible sources of myofibroblasts in the liver are portal and periductular fibroblasts after BDL (Tuchweber et al. 1996, Dranoff and Wells 2010), and smooth muscle cells and central vein fibroblasts in rats (Bhunchet and Wake 1992, Bataller and Brenner 2005, Friedman 2008, Popov and Schuppan 2009). Showing that these mesenchymal cells with a common origin share the response of transdifferentiation to myofibroblasts upon liver injury (Asahina 2012).

Platelet derived growth factor (PDGF) signalling is important in HSCs and is involved in their migration, proliferation and differentiation to a myofibroblast phenotype (Friedman and Arthur 1989). In models of biliary damage it has a role in fibrogenesis. BDL leads to the formation of reactive cholangiocytes which express increased amount of PDGFβ (Grappone et al. 1999). This causes the chemo-attraction of HSCs (Kinnman et al. 2000), which express PDGF receptor β (PDGFRβ), to the ducts and also their proliferation followed by differentiation into myofibroblasts (Kinnman et al. 2001). PDGFβ has also been shown to control cholangiocyte proliferation after biliary injury (Omenetti et al.
2008). This highlights the roles of non-parenchymal cells in liver disease and the importance of the interactions between them.

NCAM is expressed in adult cirrhotic and fibrotic livers, even though its expression is lost during development, and so maybe an activation marker (Cassiman et al. 2002). HSC are known to express NCAM on their surface and in their cytoplasmic projections, staining is weaker in the central vein area of the liver and absent in the SMCs, portal fibroblasts and central vein myofibroblasts (Knittel et al. 1996). GFAP is also expressed in HSCs (Gard et al. 1985, Neubauer et al. 1996). Meaning these markers can be used to distinguish between the different cell populations. HSC and myofibroblasts in human adult cirrhotic liver were αSMA+/NCAM+/DES-ve. The SMC were DES+/αSMA+/NCAM-ve/GFAP-ve. Portal and septal myofibroblasts were DES-ve/αSMA+/NCAM-ve/GFAP+ (Cassiman et al. 2002).

HSCs are a source of myofibroblasts in hepatotoxic liver fibrosis (Friedman et al. 1985). In mice after 2 months CCl4 treatment, 98% of COL1 expressing cells co-expressed DES and 94% co-expressed αSMA. CCl4 treatment also induces proliferation of HSCs with their numbers going from 10% of the cells of the uninjured liver to 14% after treatment. In mice qHSCs are GFAP+/DES+/SMA-/COL1-ve whereas aHSCs are GFAP+/DES+/SMA+/COL1+ (Kisseleva et al. 2012).

In vitro work has shown that aHSCs can regain a quiescent phenotype (She et al. 2005) becoming inactive HSCs (iHSC). With αSMA myofibroblasts undetectable 1 month after injury (Kisseleva et al. 2012). This iHSC phenotype is similar to but distinct from their original phenotype and has also been demonstrated in vivo in mice using COL1-GFP (Kisseleva et al. 2012).

COL-GFP mice have also been used to show that HSCs transiently express COL1 during development between E16.5 and P14, without becoming myofibroblasts (Kisseleva et al. 2012). Coupled with their transient αSMA expression it shows fetal HSCs temporarily adopt aHSC markers. However this process must be different during development than in injury, as fetal HSCs and qHSCs can still be distinguished from iHSCs in their lower expression of GFAP, ADFP and Adipor1 (She et al. 2005, Kisseleva et al. 2012).

1.2.4 Vasculature of the Liver

In the adult there is a dual blood supply into the liver from the portal vein and hepatic artery, and a single out flow through the central vein (Matsumoto and Kawakami 1982, Lalor et al. 2006). Connecting the two are the hepatic sinusoids, a microvasculature system
specific to the liver which take the mixed portal blood supply via the hepatocytes and out to the central vein (Fig. 6). The hepatic artery gives rise to capillary branches associated with bile ducts known as the peribiliary plexus (PBP). The PBP has both efferent and afferent vessels which wrap around the duct, it drains into both the sinusoid and portal vein branches (Takasaki and Hano 2001).

**Figure 1-6 The vasculature of the liver**

A diagram showing the vasculature organisation of a liver lobule. Adapted from: Anatomy & Physiology, http://cnx.org/content/col11496/1.6/, OpenStax College.

During human development the portal vein is derived from the vitelline veins of the embryo at 6wpc (Gouysse et al. 2002, Collardeau-Frachon and Scoazec 2008), this precedes the development of the hepatic artery which starts at 8wpc and reaches the liver periphery at roughly 15wpc. The central veins can first be identified around 10wpc (Gouysse et al. 2002).

Vasculogenesis of EC precursors within the STM leads to the formation of capillaries. These go on to form the sinusoids of the liver through angiogenesis, after the invasion of the STM by the nascent liver bud (Couvelard et al. 1996, Collardeau-Frachon and Scoazec 2008). Lineage tracing has shown that the mesothelium is the origin of the hepatic sinusoidal cells in the avian liver (Perez-Pomares et al. 2004) which is in contrast to the lack of MESP1 derived LSECs seen in the mouse models.

The hepatic artery develops via the process of angiogenesis, the extension of existing vessels, starting at the hilar and spreading out to the periphery. This matches the pattern of bile duct development and occurs in tandem with the differentiation of the ductal plate, both of which are preceded by the branches of the portal vein (Gouysse et al. 2002). The
hepatic artery development may be driven by the vascular endothelial growth factor (VEGF) production of the ductal plate (Si-Tayeb et al. 2010).

The PBP is thought to develop from mesenchymal precursors through vasculogenesis (de novo formation rather than extension of existing vessels), this is further supported by the finding of isolated CD34+ cells, potential endothelial precursors known as angioblasts, in the portal areas prior to the formation of the capillaries (Nakanuma et al. 1997, Gouysse et al. 2002).

Gouysse et al show both sinusoids and portal veins undergo differentiation during development involving changes in endothelial markers and ECM proteins. Laminin expression varies in the different endothelial compartments. Arteries, efferent veins and portal capillaries were found to be in contact with laminin (LAM) throughout development whereas sinusoids were only LAM+ until 10wpc. In the portal vein laminin was undetected until 10wpc after which it increased along the portal vein branches (Gouysse et al. 2002).

Expression of CD34, an endothelial cell marker, also varies with development and EC function. Portal capillaries are always CD34+ whereas in sinusoids this expression is lost between 8-10wpc and portal veins gradually lose expression between 10 and 15wpc. Central veins progressively lost CD34 expression after 20wpc and it was never expressed by the arteries (Gouysse et al. 2002).

The markers of the sinusoids at 5-10wpc remain those of capillaries including continuous cell junctions and CD31 expression. CD31 is undetectable after 10wpc and specific sinusoidal markers become detectable after 15wpc (Gouysse et al. 2002). While the features of the fully differentiated sinusoids such as fenestrations are not seen until 17wpc (Enzan et al. 1983).

This is in contrast to data from Pusztaszeri et al which shows CD34 staining in periportal sinusoids/capillaries, and venous and arterial ECs as well as CD31 staining in all the ECs of the liver (Pusztaszeri et al. 2006). The reason for this discrepancy could be the use of non-fetal tissue of different ages, with Gouysse et al’s oldest tissue being P5 whereas Pusztaszeri et al use tissue biopsy of unspecified age. The sinusoids are known to undergo capillariization with advanced age which could lead to older samples expressing more CD34 and CD31 (Le Couteur et al. 2005).

After 25wpc the architecture and markers of the liver vasculature showed no further changes. This period of vasculature organization matches the period of maximum VEGF expression (5-25wpc) by the fetal hepatocytes, after which its expression is restricted to hepatocytes surrounding portal tracts (Gouysse et al. 2002). VEGF was found in all hepatocytes from 5-15wpc. Between 15-25wpc this decreased, except around the ductal
plate, beginning with the central vein area. No VEGF staining of differentiating biliary epithelial cells (BEC) or remodelling bile ducts was found at any stage of development (Gouysse et al. 2002) which is in contrast to the Fabris et al data (Fabris et al. 2008). They also found it was undetectable in hepatocytes from 40wks and immediately postnatally (Fabris et al. 2008).

1.2.4.1 LSECs

LSECs line the sinusoids and interface the circulation with the hepatocytes. They are highly endocytotic (Smedsrod et al. 1990) and involved in both the development (Matsumoto et al. 2001) and regeneration (LeCouter et al. 2003) of the liver. Interactions between the vasculature and the developing endoderm are essential for hepatic specification (Si-Tayeb et al. 2010).

In the adult liver LSECs make up 2.5% of the parenchyma, they surround the single cell cords of hepatocytes on their basolateral surface and are found in close association with the hepatic stellate cells (Si-Tayeb et al. 2010).

After their acquisition of a fenestrated phenotype the LSECs remain distinct from the other endothelial cells of the liver but do share features with the sinusoidal endothelium found in the spleen and bone marrow, such as a minimal basement membrane and loose cell junctions. Liver sinusoids show some differences however, they are closely associated with Kupffer cells and have open fenestrations arranged in sieve plates (DeLeve et al. 2004) (Lalor et al. 2006).

The LSECs produce a discontinuous type IV collagen basement membrane (Hahn et al. 1980) sparsely distributed along their length (Sarin 2010). Fenestrations are 50-150nm in diameter and the sieve plates consist of groups of 10-100 fenestrations (Svistounov et al. 2012). These allow the transfer of larger molecules between the hepatocytes and the bloodstream such as hormones and albumin (Si-Tayeb et al. 2010).

LSECs have their own specific functions, as well as those shared by all ECs, such as acting as barrier and filter between the blood supply and the liver parenchyma (Lalor et al. 2006). Together with their associated Kupffer cells the LSECs act as a scavenger system (Elvevold et al. 2004) using scavenger receptors to bind and transfer materials between the liver and the blood supply which is part of both the gut and systemic circulation (Lalor et al. 2006). Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) is one such scavenger receptor and part of the link family of proteins (Lalor et al. 2006). It is also expressed in the lymphatic ECs and is thought to be involved in hyaluronic acid uptake, as
an endocytotic receptor, and leukocyte adhesion (Banerji et al. 1999). It is expressed in both fetal and adult LSECs (Mouta Carreira et al. 2001, Morisada et al. 2005) and in adult liver its expression varies with zonation, with the highest expression seen in zone 2 (Lalor et al. 2006). LSECs have also been found to express VAP1, L-sign, stabilin-1 (STAB-1) and its homologue STAB-2 both hyaluronan receptors, and low levels of CD31/PECAM in comparison to veinous endothelial cells (Lalor et al. 2006).

At E9.5 when the hepatoblasts invade the STM its endothelial cells are CD31+, these are the cells which give rise to the sinusoids (Asahina et al. 2011). At this point fetal liver kinase-1 (FLK-1) positive cells are seen in the vitelline vein and liver parenchyma (Nonaka et al. 2007), they have been shown to promote hepatic growth, morphogenesis and differentiation (Matsumoto et al. 2001, Lammert et al. 2003). Some FLK-1+ cells co-expressed STAB-2 in the parenchyma, acquiring their liver specific phenotype, but were LYVE1-ve. At E9.75 lumen formation was seen in FLK-1/STAB-2 coexpressing cells (Nonaka et al. 2007). At E10.5 STAB-2 is expressed in both sinusoids and veins while LYVE1 expression begins in a few sinusoids. At E14.5 LYVE1 expression has increased and by E17.5 it is found in all the sinusoids, STAB-1 and LYVE1 are then both continuously expressed (Nonaka et al. 2007).

From E9.5-14.5 CD34 is expressed (Nonaka et al. 2007) at which point there are no fenestrations (Enzan et al. 1997, Nonaka et al. 2007). From E15.5 onwards fenestrae form and the cell junctions become discontinuous (Enzan et al. 1997) while CD34 expression decreases (Nonaka et al. 2007). Showing that LSECs have acquired their tissue specific features by the mid-stage of liver development defined as 10wpc in human, E15 in mice and rat (Nonaka et al. 2007). FACS analysis showed 95% of CD31+ ECs in the fetal liver expressed LYVE1 and 79% in the adult. Fetal LSECs at E14.5 were found to have a higher endocytotic activity than blood ECs or lymph ECs showing LSEC specific function early in development (Nonaka et al. 2007).

1.2.4.2 Capillarization

Human liver vasculature is developed by 17-25wks and becomes fenestrated by 20wpc (Gouysse et al. 2002). Macromolecules in the blood can pass through the fenestrations to interact with the hepatocytes (Steffan et al. 1995). Fenestrations can cover up to 40% of the LSEC and their size and distribution can vary in the different zones of the liver (Vidal-Vanaclocha and Barbera-Guillem 1985).
During ageing and disease the number of fenestrations decreases caused by dedifferentiation of the LSECs in a process known as capillarization (Le Couteur et al. 2001, Le Couteur et al. 2005). This is associated with a reduction in fenestrae and scavenger behaviour, changes in the basement membrane and an increase in CD31 expression (DeLeve et al. 2004) and may prevent the movement of molecules between the blood and the hepatocytes and also cause hypoxia. Cirrhosis and hepatitis can also cause capillarization (Xu et al. 2003).

When LSECs are isolated and cultured in vitro their morphology is altered, becoming flattened, rapidly losing fenestrae and undergoing capillarization (Lalor et al. 2006). Fenestrations can be maintained and capillarization prevented by the addition of VEGF or by culture on collagen (Funyu et al. 2001, Yokomori et al. 2003, DeLeve et al. 2004) without which they are lost within 48hr (Funyu et al. 2001).

CD34 is a type 1 transmembrane sialomucin expressed on hematopoietic stem cells and microvasculature with a continuous endothelium such as capillaries and lymph vessels but LSECs are negative in the adult liver (Scoazec and Feldmann 1991, Couvelard et al. 1993, Daneker et al. 1998, Xu et al. 2003). Its expression increases during disease and capillarization (Cui et al. 1996, Frachon et al. 2001).

CD31 is a membrane protein involved in cell adhesion, expressed on ECs and haematopoietic cells. It is used as a marker of continuous vasculature and also found by some groups to be a marker of LSECs (Do et al. 1999, Neubauer et al. 2000) but described as absent by others (Scoazec and Feldmann 1994, Knolle et al. 1999). In the human embryo LSEC are CD31-ve until 25wpc. It is found in the normal adult liver but at lower levels than in vascular ECs (DeLeve et al. 2004, Lalor et al. 2006) with expression increased in diseased liver (Daneker et al. 1998, Medina et al. 2005, Lalor et al. 2006). CD31 is also present on Kupffer cells in the sinusoid. Some researchers have used the specific cellular localisation of CD31 as a sign of dedifferentiation (DeLeve et al. 2004), interpreting cytoplasmic expression as normal with dedifferentiated cells showing increased membrane expression.

CD31 is not expressed in the LSEC of adult mice. Walter et al found capillarization in the LSECs of VEGF KO mice with increased CD31 and laminin expression. In control adult mice laminin marks the vasculature and the ducts with small amounts visible in the sinusoidal spaces, this sinusoidal staining is greatly increased in the VEGF KO (VKO) mice especially in pericentral (PC) regions (Walter et al. 2014).

Staining of laminin in human liver shows it is found throughout the liver until 10wpc, after which it is lost, except in the central vein areas. It is absent in portal areas until 10wpc and
then increases (Gouysse et al. 2002). Expression of laminin is linked with the continuous EC markers in the early LSECs and in capillarization. The LSECs contact with the ECM may determine its phenotype, with laminin associated with a continuous endothelium and COLIV associated with a discontinuous fenestrated endothelium.

1.2.4.3 Interactions between ECs and other hepatic cells

Both in vivo and in vitro experiments have been used to reveal the cross talk between the endothelial cells and the other liver cell types. PDGFβ induces a pericyte phenotype in HSCs leading to their coverage of the sinusoids and promoting vasculature morphogenesis (Semela et al. 2008). ECs use PDGFβ to recruit the PDGFRβ expressing pericytes, which are involved in the formation of the vasculature (Hellstrom et al. 1999). Showing the interaction with the pericytes/HSCs plays a role in the formation of structure from the ECs. WNT signalling has also been shown to be involved in the proliferation and differentiation of LSECs (Matsumoto et al. 2008). ECs promote the growth of hepatic progenitor cells in early mouse liver buds (E9.5), prior to the formation of functional vessels, indicating that they play a role in the development of the parenchyma. This signalling was dependent on WNT9a (Matsumoto et al. 2008).

WNT2 is expressed by rat LSECs and via β-catenin signalling increases their proliferation. A decrease in WNT2 lead to a decrease in VEGF receptor 2 (VEGFR2) showing crosstalk between the WNT and VEGF pathways (Klein et al. 2008). VEGF treatment of a hepatocyte LSEC co-culture caused secretion of HGF and Interleukin-6 (IL-6), amongst other mitogens, by the LSECs which in turn leads to increased proliferation of the hepatocytes (LeCouter et al. 2003). These interactions between the LSECs and parenchyma show that their development is dependent on each other.

Vasculogenesis, the proliferation and differentiation of ECs is driven by VEGF, whereas angiogenesis, the growth and remodelling of existing vessels, is regulated by angiopoietins. VEGF and VEGF receptors are both expressed in many cell types found within the liver, including; hematopoietic stem cells, ECs, cholangiocytes and HSCs (Morell et al. 2013). VEGF is secreted by both hepatocytes and cholangiocytes during regeneration after injury (Ishikawa et al. 1999, Mancinelli et al. 2009), and induces the proliferation of LSECs (Shimizu et al. 2001), showing that VEGF allows communication between hepatocytes, cholangiocytes and ECs. Complete blocking of VEGF signalling in mice has been shown to affect the organisation of hepatocytes and sinusoids and reduces the number of LSECs (Gerber et al. 1999, Carpenter et al. 2005).
VEGF signalling links the formation of the bile ducts and the liver vasculature. Fabris et al showed that there is a VEGF gradient produced by the developing bile duct, beginning at the onset of ductal plate formation and increasing during development. The secreted VEGFA acts on ECs to induce formation of the arteries and the PBP. Pericytes were then recruited to the vessels by the secretion of ANG-1 from the hepatoblasts (Fabris et al. 2008). Hepatic artery ligation in rats causes a decrease in VEGFA expression and reduced proliferation in cholangiocytes and loss of the PBP. These effects were reversed by administration of rVEGF, highlighting its importance in both ductular and vasculature structures (Gaudio et al. 2006). In polycystic liver disease the cholangiocytes form large cysts/spheroids throughout the liver. These consist of immature proliferative cholangiocytes and are surrounded by extensive vasculature (Morell et al. 2013). In these diseased livers VEGF was shown to induce both the proliferation of the cholangiocytes and their associated vasculature in a dose dependant manner (Fabris et al. 2006). Showing VEGFs role in cholangiocyte and EC proliferation.

Walter et al used a model of reduced VEGF liver signalling, by specifically knocking out VEGF in the hepatoblasts and their descendants. They found parenchymal VEGF expression was necessary for correct development of the vascular architecture and hepatocyte zonation (Walter et al. 2014). VEGF secretion peaks during development, at E16.5, and continues to drop postnatally. Liver specific VKO mice have reduced VEGF in the liver showing it is secreted from hepatoblasts and or their descendants in the wild type (WT) mice. Initially this loss is not reflected in the serum but during development in VKO mice VEGF serum levels decrease compared to the WT (Walter et al. 2014). Suggesting that in the WT mice the liver secretes an increasing proportion of the total circulatory VEGF during development. At P30 the VKO mice had dilated sinusoids and areas of necrosis (Walter et al. 2014) showing parenchymal VEGF secretion is necessary for correct development of the vascular architecture. During normal development there is an increase in portal branching between P15 and P30 but at P30 in VKO mice there is a reduction in portal vein branching. This pattern was also found to exist in the hepatic artery (Walter et al. 2014).

GS is a PC hepatocyte marker involved in glutamine formation. While carbamoyl phosphate synthase 1 (CPS1) is found in zone 1 (periportal) and zone 2 hepatocytes and functions in urea formation (Jungermann and Kietzmann 1996). GS expression, normally restricted to central vein surrounding hepatocytes, was found in large clusters of non-PC hepatocytes of the VKO mice by P30. This expanded GS staining is of a reduced intensity and includes even periportal (PP) hepatocytes. At these stages CPS1 and GS co-expression
is seen which is absent from control mouse livers, with some CPS1+ cells also found in PC areas. The authors conclude that the altered gene expression indicates a loss of zonation and the hepatocyte phenotypes specific to it (Walter et al. 2014). Use of a hypoxia stain shows a slight increase in hypoxia in VKO livers postnatally. In both KO and control hypoxia is greater at P15 than P30 and appears to map roughly to the strongest areas of GS expression. This is consistent with the different liver zones being exposed to different amounts of oxygen (Walter et al. 2014).

Walter shows that abnormal zonation and expanded expression of GS are linked with areas of increased hypoxia which in turn may be linked to the changes (capillarization and reduced portal and artery branching, less extensive PBP) seen in the vasculature system caused by the VKO (Walter et al. 2014). GS is normally regulated by HNF4α and WNT/β-catenin (Cadoret et al. 2002) this signalling may be disrupted by the hypoxia leading to aberrant GS expression in non-PC hepatocytes.

While Bile ducts were not visibly affected in VKO mice, this does not directly contradict the Fabris paper which states that the ductal plate drives hepatic artery and PBP formation not the other way round, and these things are affected in the VKO mice. There is also still VEGF in the system which may be enough to satisfy any BEC requirement. VKO mice show abnormalities in all there vasculature systems, but these occur in different systems at different times. Walter et al hypothesise a different dose of VEGF is required for different vasculature systems and that the decreasing VEGF of the VKO mice drops below these thresholds at different times. With higher amounts needed for arteries and microvasculature and lower amounts needed for veins (Walter et al. 2014). Hence the most dramatic effect on those systems with the highest requirements. An alternative explanation for the disruptions is suggested to be spatially localised VEGF signalling.

1.2.5 Cholangiocytes and Bile ducts

1.2.5.1 Development of the Biliary System

The apical surfaces of adjacent hepatocytes form channels known as canaliculi. Bile acids, produced from cholesterol in the hepatocytes (Russell and Setchell 1992), and salts are secreted into these canaliculi; then flow into the bile ducts and to the intestine where they emulsify fat (Si-Tayeb et al. 2010). The biliary system contains both intra and extra hepatic structures both lined with cholangiocytes. The BECs of the intra hepatic bile ducts (IHBD) make up roughly 3% of the adult liver cell population (Si-Tayeb et al. 2010) and drain the canaliculi. Bile flows from the IHBDs of the liver lobes into the hepatic duct, then to the
gallbladder in the cystic duct, and finally to the gut through the common bile duct, these collectively form the extra hepatic biliary ducts (EHBD) (Clotman et al. 2002).

The Liver, EHBD and pancreas all develop from the posterior ventral foregut. The EHBDs arise directly from the ventral endoderm. They are formed from precursors also capable of differentiating into the pancreas which are PDX1+/SOX17+. These cells separate into a SOX17+/PDX1- cholangiocyte population and a SOX17-ve/PDX1+ pancreatic one (Spence et al. 2009). Overexpression of SOX17 in the PDX1+ cells prevents pancreas formation (Spence et al. 2009). Hairy and enhancer of split 1 (HES-1) is upstream of SOX17 and in HES-1 KO mice EHBD cells instead differentiate into pancreas cells (Sumazaki et al. 2004, Fukuda et al. 2006). Cholangiocytes of the IHBD, in contrast, have a distinct origin and are derived from the bipotential hepatoblast of the developing liver parenchyma (Lemaigre and Zaret 2004, Spence et al. 2009).

Expression from the shared AFP/ALB promoter occurs in the hepatoblasts prior to cholangiocyte differentiation (Coffinier et al. 2002), however the BEC do not express albumin (Shiojiri 1997). The gallbladder also shows expression from the AFP/ALB promoter but in a heterogeneous pattern, showing that it derives in part from hepatic specified cells, this is seen at E10.5 in the mouse liver bud (Coffinier et al. 2002).

1.2.5.2 IHBD formation and morphogenesis - Transient asymmetry in IHBD

Portal vein branches are the sites of duct formation for IHBDs. The formation of the biliary ducts from the bipotent hepatoblasts requires several distinct stages. First the hepatoblasts adjacent to the portal mesenchyme differentiate to cholangiocytes, forming rings around the portal vein known as the ductal plate (Fig. 7, panel 1) (Shiojiri and Katayama 1987, Clotman et al. 2002, Lemaigre 2003). The ductal plate then becomes bilayered but not due to ductal plate cell proliferation (Tanimizu et al. 2009) instead lumens form at discrete locations between the cholangiocytes and their adjacent hepatoblasts, resulting in asymmetrical ductal structures (Fig. 7, panel 2) (Antoniou et al. 2009). The lumen surrounding hepatoblasts next differentiate to cholangiocytes while the remaining ductal plate cells, which do not surround a lumen, lose their cholangiocyte markers (Fig. 7, panel 4). The bilayers of the asymmetrical duct show transient asymmetry in the markers they express. The ductal plate on the portal side of the lumen expresses the biliary markers; SOX9, and cytokeratin 19 (CK19). While the second layer of cells on the parenchymal side of the lumen expresses HNF4α, characteristic of hepatoblasts (Fig. 7, panel 2 and 3). This asymmetry is resolved as the duct matures and all the cells surrounding the lumen express
cholangiocyte markers (Fig. 7, panel 4) (Ito et al. 2007, Antoniou et al. 2009, Zong et al. 2009).

Figure 1-7 IHBD formation and morphogenesis

Section 1 shows the portal vein surrounded by the ductal plate which in turn is surrounded by hepatoblasts. Section 2 shows the formation of lumen and asymmetrical duct structures. Section 3 shows a PDS which still expressing markers of both hepatocytes and cholangiocytes. Section 4 shows a mature bile duct fully surrounded by the portal mesenchyme, the regression of the ductal plate and the differentiation of hepatoblasts to hepatocytes. Figure from “Organogenesis and development of the liver” (Si-Tayeb et al. 2010).

Cell polarity also develops radially in the primitive duct structure (PDS). At the beginning of PDS formation all ductal plate cells display the apical pole marker osteopontin (OPN), while cells in contact with laminin on the portal side of the PDS are positive for the basal marker E-cadherin. This basal polarity is acquired by the cells on the parenchymal side as
they too come into contact with laminin and the PDS matures, as shown by increased E-cadherin expression (Antoniou et al. 2009).

As well as ducts maturing sequentially along a radial axis, they also mature on an axis that runs in their direction of growth from the hilum to the periphery of the liver. This results in ductal cells near the hilum achieving radial symmetry whilst ductal cells on the periphery may still be asymmetrical (with respect to marker expression) and maturing, dependant on the stage of development. In fully developed livers all ducts display radial symmetry (Van Eyken et al. 1988, Antoniou et al. 2009).

Cholangiocyte proliferation occurs after formation of symmetrical ducts in vivo, beginning at the end of gestation (Si-Tayeb et al. 2010). This proliferation is controlled by their primary cilia which are osmo-, chemo- and mechano-sensitive (Masyuk et al. 2008).

### 1.2.5.3 Signalling pathways

#### 1.2.5.3.1 Notch signalling

The signalling pathways thought to be involved in localising cholangiocyte formation to the portal area are transforming growth factor β (TGFβ) and Notch. Notch signalling is thought to control the initial differentiation of hepatoblasts to cholangiocytes as liver specific mice KOs of RBP-Jκ, a Notch pathway protein, reduce the number of cholangiocytes formed (Zong et al. 2009). Tanimizu et al. showed Notch signalling promotes the biliary fate and represses hepatocyte specification in hepatoblasts in vitro (Tanimizu and Miyajima 2004).

Alagille syndrome causes defects in bile duct development; it results from mutations in JAG1 or NOTCH2 suggesting Notch signalling plays a role in biliary development (Oda et al. 1997, McDaniell et al. 2006). JAG1 is expressed in the periportal mesenchyme and, along with its receptor NOTCH2, in the adjacent differentiating cholangiocytes (Flynn et al. 2004, Geisler et al. 2008, Zong et al. 2009). Notch signalling requires cell to cell contact with the JAG1 expression spreading from the ECs of the portal vein to the mesenchyme and finally to the biliary precursor cells (Louis et al. 1999, Loomes et al. 2002, McCright et al. 2002, Flynn et al. 2004, Kodama et al. 2004, Tanimizu and Miyajima 2004, Zong et al. 2009). This spread of Notch signalling from the cells which constitute the portal area may be responsible for the biliary differentiation of hepatoblasts and its restriction to a specific area (Loomes et al. 2002, McCright et al. 2002, Kodama et al. 2004, Tanimizu and Miyajima 2004).
JAG1 and HES-1, another protein of the Notch signalling pathway, both follow a sequential expression pattern in the development of the PDS, being first expressed on the portal side of the ductal plate followed by the parenchymal (Fig. 6, panels 2-4), suggesting Notch signalling from the ductal plate causes the adjacent hepatoblasts to also become biliary cells (Zong et al. 2009). Knockouts of HES-1 showed it was necessary for the formation of the PDS from the ductal plate (Kodama et al. 2004). In vivo HES-1 expression in the second layer of biliary cells precedes the expression of biliary markers.

1.2.5.3.2 TGFβ signalling

TGFβ signalling originates in the periportal mesenchyme (Raynaud et al. 2011). Within the liver it forms a gradient with the highest activity in the portal area, where ductal plate formation occurs, and lower levels in the parenchyma (Clotman et al. 2005, Antoniou et al. 2009). Disruption of this gradient leads to high TGFβ signalling throughout the parenchyma causing the hepatoblasts to undergo differentiation resulting in hybrid cells with a mixed hepatobiliary phenotype (Clotman et al. 2005). The transcription factors HNF6 and OC-2 cause the localisation of high levels of TGFβ to the periportal region. In double knockouts of HNF6 and OC-2, TGFβ signalling is detected throughout the liver (Clotman et al. 2005). Leading to the conclusion that TGFβ signalling is involved in biliary differentiation. (Clotman et al. 2005, Clotman and Lemaigre 2006). In vivo experiments have also shown that anti-TGFβ antibodies can block biliary differentiation (Clotman et al. 2005).

Transient expression of the TGFβ receptor 2 (TGFBR2) is seen in the ductal plate, before being lost again in the cholangiocyte suggesting it also plays a role in biliary differentiation (Antoniou et al. 2009). TGFBR2 expression follows the pattern of cholangiocyte differentiation during bilayered ductal plate formation and asymmetric PDS maturation. The single layered cholangiocyte precursors of the ductal plate express TGFBR2. This expression is lost in the cells on the portal side of the PDS as they differentiate to cholangiocytes, but gained in the second parenchymal layer of hepatoblast like cells (Antoniou et al. 2009). These cells in turn stop expressing TGFBR2 as the ducts mature and radial symmetry is achieved, leaving ducts lined with cholangiocytes (Antoniou et al. 2009). TGFβ signalling not only promotes cholangiocyte differentiation but also represses TGFBR2 expression (Antoniou et al. 2009).

Another mechanism which affects the TGFβ gradient is the spatially different expression of its isoforms. TGFβ-1 is expressed throughout the parenchyma whilst TGFβ-2, TGFβ-3 and activin A are localised to the periportal region, the site of cholangiocyte differentiation.
1.2.5.3.3 WNT signalling

WNT signalling via β-catenin is involved in cholangiocyte specification. β-catenin expression changes with time during hepatoblast differentiation, peaking at E12.5 and disappearing again at E16.5 (Micsenyi et al. 2004). The adenomatosis polyposis coli (APC) gene marks β-catenin for degradation, its deletion increases the activity of the WNT/β-catenin pathway and promotes biliary differentiation of hepatoblasts, whereas inactivation of β-catenin promotes hepatocyte differentiation. Showing that high WNT/β-catenin pathway activity promotes biliary differentiation of hepatoblasts (Decaens et al. 2008, Tan et al. 2008). β-catenin liver knockouts result in only a small number of primitive bile ducts developing and also cause problems with hepatoblast growth and survival (Tan et al. 2008). In ex vivo liver culture, WNT3a has been shown to induce ectopic biliary differentiation (Hussain et al. 2004).

1.2.5.3.4 ECM

In vivo the basal polarity of cholangiocytes is associated with laminin contact. Tanimizu et al (Tanimizu et al. 2007) found culturing hepatoblasts on matrigel, which contains laminin, induced expression of cholangiocyte markers and apico-basal polarity. Cholangiocytes are known to express the integrins α6β1, α2β1, α3β1 and α6β4, which are laminin receptors (Couvelard et al. 1998).

1.2.5.4 Transcription Factors in biliary differentiation

The first marker of biliary differentiation in the developing liver is the expression of the TF SOX9 (Antoniou et al. 2009). SOX9+ hepatoblasts are found as a single layer of cells surrounding the periportal mesenchyme, which itself surrounds a branch of the portal vein. It acts downstream of HNF6 in biliary development (Antoniou et al. 2009). Maturation of bile ducts, evident as a loss of the asymmetry of cholangiocyte marker expression, is delayed in SOX9 mutants (Antoniou et al. 2009). NOTCH1 can bind directly to the SOX9 promoter and its constitutive expression has been shown to cause an upregulation in SOX9 expression in vivo, which suggests SOX9 is a target of notch signalling (Zong et al. 2009).
SOX9 hepatic expression begins at E11.5 in cells near the portal vein. During liver development it is restricted to the biliary rather than hepatocyte lineage (Antoniou et al. 2009). CEBPα may also regulate biliary morphogenesis. It is expressed in hepatoblasts and hepatocytes but not the cholangiocytes of the PDS and is repressed by SOX9 (Yamasaki et al. 2006, Antoniou et al. 2009).

Lower levels of HNF4α expression and higher levels of HNF6 and HNF1β are found in differentiating cholangiocytes. Deletion of TBX3 in hepatoblasts at the liver bud stage can mimic these expression profiles (Ludtke et al. 2009).

Dual KOs of FOXA1 and FOXA2 cause cholangiocyte proliferation and in turn biliary hyperplasia through increased expression of IL-6 (Li et al. 2009). IL-6 causes cholangiocyte proliferation, it is inhibited by GR binding to its promoter but in the FOX KOs this no longer occurs.

HNF6 and HNF1β are involved in biliary development, knockouts of these transcription factors causes symptoms also found in human biliary diseases such as abnormal ductal plate and lumen formation (Clotman et al. 2002, Coffinier et al. 2002).

When both HNF6 and OC-2 are deleted, hepatoblasts form cells which express hepatocyte and cholangiocyte markers, as the dual KO relieves their inhibition of TGFβ signalling (Clotman et al. 2005). CEBPα KO mice also result in cells with a mixed hepatobiliary phenotype and abnormal duct structures (Clotman et al. 2005, Yamasaki et al. 2006).

1.2.5.4.1 HNF1β KO

HNF1β is a homeodomain transcription factor. In normal development it is expressed in the liver diverticulum (Barbacci et al. 1999, Coffinier et al. 1999), late gestation bile ducts and developing gallbladder (Coffinier et al. 1999). Adult livers express HNF1β throughout the biliary ducts (Coffinier et al. 2002).

Specific HNF1β KO in hepatocytes and cholangiocytes causes liver hypertrophy and jaundice, which indicates biliary damage and is caused by accumulation of bile acids. It also resulted in abnormal development and dysplasia of EHBDS, gallbladder and IHBDs and a paucity of small IHBDs. CK staining in post natal mice at 1wk and 2mths revealed no small biliary ducts within the portal tracts but larger ducts were found with a disorganised and multilayered epithelium (Coffinier et al. 2002). The HNF1β liver KO (LKO) resulted in persistence of the ductal plate after birth (Coffinier et al. 2002). Showing that while HNF1β is necessary to drive BEC differentiation it may not be necessary to induce it.
In the WT a CK+ ductal plate is seen at E15.5 with lumens forming at E16.5 after which the ducts are incorporated into the mesenchyme and the remaining ductal plate regresses. The ductal plate in the HNF1β LKO was irregular and had disrupted lumens. At P8 the mutant ducts are still irregular with misshapen lumens and sparse with roughly 5% of that of the WT livers (Coffinier et al. 2002).

Hepatic arteries were absent, despite a lack of HNF1β expression in these cells in the WT, highlighting the link between their formation and that of the biliary system. The lack of arteries may in turn contribute to the disorganisation of the IHBDs (Desmet 1992, Coffinier et al. 2002).

HNF1β LKO had no effect on expression levels of HNF1α, HNF4α, FOXA2, and HNF6 (Coffinier et al. 2002). In contrast to the HNF1β expression in adult BECs but not hepatocytes (Yamasaki et al. 2006), HNF1α is expressed in all hepatocytes and is low in the BECs (Pontoglio et al. 1996). While HNF1α and HNF1β can bind the same DNA sequences, different effects are seen in HNF1α and HNF1β KOs, this may due to different requirements for them as homodimers and heterodimers, meaning they do not always function redundantly (Coffinier et al. 2002).

1.2.5.4.2 HNF6 KO

HNF6 is expressed in the hepatoblasts and gallbladder, starting at the onset of their formation (Landry et al. 1997, Rausa et al. 1997) and also in the BEC of the EHBD and IHBD (Clotman et al. 2002). HNF6 mRNA expression was found in the liver continuously from E12.5-E16.5. At E15.5 HNF6 expression was heterogeneous amongst hepatocytes and expressed at higher amounts in the ductal plate, gallbladder and EHBD (Clotman et al. 2002). HNF6 KO mice lack a gall bladder and have an abnormal biliary system, both the IHBD and the EHBD, showing HNF6 is necessary for the formation of the biliary tract.

CK19 and ki67 do not co-localise at E14.5 in HNF6 KO or wild type (WT) mice showing the BECs are not proliferative at this stage. This is consistent with the proposal that these cells are non-mitotic (Fabris et al. 2000, Clotman et al. 2002). Hence the excess biliary cells and abnormal duct structures seen in developing (e13.5) HNF6 KO mice are thought to be caused by an increase in hepatoblast to cholangiocyte differentiation rather than BEC proliferation (Fabris et al. 2000, Clotman et al. 2002).

The loss of the HNF6 mediated TGFβ inhibition seen during normal development (Si-Tayeb et al. 2010) maybe what causes the hepatoblasts to differentiate to BECs in inappropriate areas in the HNF6 KO. This model maybe oversimplified as OC-2 is known to act redundantly with HNF6 (OC-1).
Expression of functional cholangiocyte markers at P3 in the HNF6 KO mice was unaffected (Clotman et al. 2002) and the disrupted duct structures expressed some markers seen in adult BECs (laminin and NCAM) but were never incorporated into the portal mesenchyme, unlike in the WT where by P10 bile ducts had completed their migration. Instead the duct like structures regressed and disappeared but no increase in apoptosis was detected (Clotman et al. 2002) suggesting they, like the ductal plate in WT mice, formed hepatocytes.

In HNF6 knockout mice laminin surrounds only the basal side of CK+ lumen lining cells but in control mice there is more laminin surrounding the CK+ cells on the apical side and in the portal mesenchyme (Clotman et al. 2002).

KO of HNF6 phenocopies the defects in IHBD development seen in HNF1β LKO fetuses, (Coffinier et al. 2002) both having disrupted duct like structures. In WT livers at E15.5 the ductal plate is strongly HNF1β positive while the rest of the parenchyma has weak heterogeneous expression. The ductal plate at this stage co-expresses both HNF1β and HNF6. Whereas at this stage in the HNF6 KOs HNF1β expression was undetected in the ductal plate but was later expressed in the BEC at E17.5 (Clotman et al. 2002) which shows HNF1β is not necessary for ductal plate formation. While HNF1β expression is heavily reduced during development in the KO it returns to normal levels postnatally (Clotman et al. 2002). This transient absence of HNF1β in the HNF6 KO may be corrected by the redundant function of OC-2. HNF6 was found to bind the HNF1β promoter suggesting that HNF6 is upstream of HNF1β (Clotman et al. 2002). HNF6 KO did not affect expression of FOXA2, FOXA3 and HNF4α during development or postnatally.

HNF6 controls the initiation of BEC differentiation, without its expression increased numbers of hepatoblasts follow a cholangiocyte fate and differentiate prematurely. HNF6 also restricts the site of BEC differentiation to the portal vein area at E13.5 (Clotman et al. 2002). Notch signalling from the portal mesenchyme is thought to induce biliary differentiation as the culture of hepatoblasts with hepatic mesenchyme causes their differentiation into BEC (Shiojiri and Koike 1997), the authors speculated that HNF6 controlled the response to this signalling (Clotman et al. 2002).

1.2.5.4.3 CEBPα KO

During cholangiocyte differentiation from periportal hepatoblasts there is down regulation of the hepatic markers; AFP, ALB, CEBPα and an increase in laminin and CKs (Shiojiri and Sugiyama 2004). CEBPα KO mice form abnormal duct like structures in non-portal parenchyma areas which express markers of both hepatocytes and biliary cells. The loss of
CEBPα suppresses hepatocyte maturation and upregulates HNF1β and HNF6 in the parenchyma (Yamasaki et al. 2006).

The differentiation of IHBD BECs in WT mice begins at E13.5-E15.5 with lumens then appearing between the first and second layers of the ductal plate. CEBPα expression was heterogeneous in the parenchyma at E12.5 and E14.5 and had increased at P0. Parenchymal cells adjacent to the portal vein are CEBPα-ve, demonstrated from E14.5 onwards; these cells will form the ductal plate. Cells with a squamous BEC morphology were visible from E16.5-17.5 (Yamasaki et al. 2006). In CEBPα KO mice lumen formation and BEC initiation occurred sooner (E12.5-E13.5), more frequently and throughout the parenchyma when compared to the WT, and resulted in pseudo duct structures. These duct like structures persist throughout development, still present at P0 and have larger lumen than normal IHBD.

Glycogen PAS staining was negative in the CEBPα KO liver at E17.5 showing a lack of hepatocyte development. There is a high amount of CK19 expression in the parenchyma of the KO at E14.5 and still at E17.5. It is restricted to the portal vein at this time point in WT livers or the BECs of the adult and could be indicative of a hepatoblast phenotype in the KO parenchyma. The cells in both WT and KO at E17.5 express HNF4α, also expressed by hepatoblasts, and show that its expression is not downstream of CEBPα. Despite the presence of these hepatoblast markers in the parenchyma of the CEBPα KO these cells are negative for ALB and AFP, and so they do not match the expression of hepatoblasts which would be HNF4α+/ALB+/AFP+ or cholangiocytes which would be HNF4α-ve/ALB-ve/ AFP-ve, and so can be described as hybrid hepatobiliary cells. This combined with the abundance of lumen formation suggests these cells have started on a cholangiocyte differentiation, with a phenotype similar to that of the second layer of the bilayered ductal plate stage of BEC formation. This could be caused by the lack of contact with the portal mesenchyme or other cholangiocytes and hence the signalling necessary to finish differentiation (Yamasaki et al. 2006). These hybrid cells show that a lack of the signalling found in the in vivo parenchyma during development leads to less than optimal production of cholangiocytes and hepatocytes, as the hepatoblasts do not properly resolve into either cell type.

CEBPα KO induction of duct like structures surrounding lumen, which resemble BEC precursors, agrees with the finding that CEBPα down regulation is necessary for IHBD formation (Shiojiri and Sugiyama 2004). However it was found that only portal and capsule cells in the KO went on to gain further characteristics of WT BECs such as; DBA, SBA, nidogen, and laminin expression (seen from E17.5), whilst being albumin, AFP and
HNF4a-ve. This shows that while CEBPα down regulation/inhibition is necessary for BEC differentiation it is not sufficient (Yamasaki et al. 2006). DBA and SBA appear to be markers of more developed ducts structures than those marked by CK19 alone as their expression occurs later in WT BECs (Yamasaki et al. 2006).

Laminin and Nidogen staining revealed higher levels in portal and capsular areas in both WT and KO livers suggesting a possible role for ECM in the cholangiocyte differentiation in CEBPα-ve cells, as only pseudoductular structures located on the ECM basement membrane found around the portal vein and the hepatic capsule form the more developed duct structures. Laminin staining shows it is not only in contact with the cholangiocytes but has small tendrils extending slightly further from the portal vein, and the hepatic capsule covers hepatocytes, so the presence of some laminin is not sufficient to cause cholangiocyte differentiation but it may be necessary. A similar role for laminin in promoting cholangiocyte differentiation was found in a mouse liver injury model (Espanol-Suner et al. 2012).

The duct structures beneath the hepatic capsule of KO livers (E17.5), absent in the WT, reveal that in the CEBPα-ve cells contact with the capsular environment is enough to induce a differentiated BEC phenotype. The presence of these structures at both portal and capsule locations reveals the presence of shared BEC inductive signalling in these areas and suggests that a factor common to both areas is necessary for BEC differentiation from CEBPα-ve cells. It also reveals that the cells at the edge of the WT liver are different from the periportal ones in their cholangiocyte forming potential but that this is not the case in the KO, meaning a lack of CEBPα expression is a pre-requisite for BEC formation.

A TGFβ gradient has been shown to be necessary to differentiate BECs from hepatoblasts which is modulated by onecut-1 (OC-1) and OC-2 (Clotman et al. 2005). This gradient with highest levels in the portal area cannot be the factor responsible for BEC differentiation in CEBPα-ve cells found in the capsular areas, and thus is also unnecessary in CEBPα-ve cells in the portal area. It may however be responsible for the initial loss of CEBPα expression in the ductal plate in WT mice in vivo.

To directly investigate the role of TGFβ, CEBPα KO E12.5 liver fragments were cultured in TGFβ1 in vitro. The authors show TGFβ was insufficient to generate cholangiocytes from hepatoblasts in both WT and KO livers. WT explants had parenchymal cells with large lumen and CK expression but no BEC markers such as DBA and PNA. These results are similar to the parenchymal cells of the in vivo CEBPα KO with their pseudo duct structures and prolonged CK expression. This is consistent with TGFβ being the CEBPα inhibiting portal signal in vivo and not the BEC inducing signal found in portal and capsule
regions, and therefore having the equivalent effect as knocking out CEBPα. Thus in the CEBPα KO TGFβ signalling would be redundant explaining why there was no difference between the WT and KO explants when treated with TGFβ1 (Yamasaki et al. 2006).

Mesenchymal cells and ECM are both present in portal and capsular locations. The portal tracts are known as Glissonian sheaths and the outer layer of the liver as Glisson’s capsule. The portal tracts can be viewed as an intrusion of this outer layer of mesenchyme derived cells and matrix into the parenchyma of the liver, as they are continuous and have a shared origin. These similarities could explain the shared ability of these areas to induce cholangiocyte differentiation in CEBPα KO cells (Yamasaki et al. 2006).

In WT liver the BEC initiation signal is portal unique, causing ductal plate formation, and is mimicked here by CEBPα KO, suggesting that it is CEBPα inhibition in vivo. In the portal position and the periphery of the KO, as opposed to the parenchyma, these duct structures acquire BEC markers. The peripheral formation of BECs in the CEBPα KO shows there is a shared factor, with the portal area, sufficient to induce primed cells to form BEC. But their absence at the periphery in WT livers shows the peripheral signalling alone is not sufficient and that a CEBPα inhibiting step is also required. Together suggesting BEC differentiation is a two stage process first requiring CEBPα suppression and then a second signal, found in both portal and capsular areas. In WT liver development the co-localisation of the BEC inducing and CEBPα inhibiting factors only occurs in portal areas, resulting in WT IHBD. The regression of some ductal plate cells back to hepatocytes after being CEBPα suppressed in vivo shows this process and the associated changes to cell phenotype must be reversible.

Periportal BECs of the KO liver were never surrounded by the portal mesenchyme unlike the WT. They had a columnar morphology, expressed markers of the EHBD from an early stage (α3 and β4 integrins) and surrounded larger lumens. Whereas in the WT IHBD, the cells are cuboidal, surround small lumen, and are β4 and DBA low and α3-ve (at E17.5) (Shiojiri and Sugiyama 2004, Yamasaki et al. 2006). Lack of IHBD characteristics in the KO means CEBPα must be having a paracrine effect on the developing ducts, as it is not required in the BECs themselves in the WT, showing IHBD development may also require concurrent hepatocyte maturation (Yamasaki et al. 2006).

In the WT as the IHBD develop they begin to express markers already expressed by the EHBD (Yamasaki et al. 2006). The EHBD are not in contact with CEBPα expressing cells during their development and since they originate directly from the endoderm have never expressed hepatoblast markers (Shiojiri and Sugiyama 2004). These differences appear to
delay the expression of BEC markers in IHBD when compared to EHBD and result in morphologically different structures.

CEBPα can inhibit HNF6 expression by binding of its regulatory sequence (Rastegar et al. 2000). HNF6 and HNF1β mRNA were detected at low levels in the WT liver parenchyma at E12.5 when compared to the EHBD. At E15.5 high expression was seen in both the forming IHBD and EHBD. From this point IHBD expression decreased while hepatocyte expression was low at E17.5 and absent at P0 (Yamasaki et al. 2006). This higher earlier expression of HNF6 and HNF1β in the EHBD in WT fits with the IHBD of the KO taking on EHBD characteristics, as the KO of CEBPα would be expected to increase the HNF6 levels in the BEC giving them EHBD levels of expression regardless of location.

In the KO HNF6 and HNF1β expression were higher than WT in the parenchyma and was highest in portal areas. At E15.5 and E17.5 this expression level remained in the parenchyma whilst increasing in the portal area and at the hepatic capsule to the levels seen in the EHBD. At P0 portal BECs were dual positive while the parenchyma was heterogeneous (Yamasaki et al. 2006). The data shows signalling in portal and peripheral areas increases HNF6 and HNF1β expression and that this correlates with BEC differentiation. Whilst all cells were HNF6 and HNF1β positive in the KO only peripheral and portal ones form BECs. This may indicate these HNFs drive a BEC phenotype in a dose dependant manner from low expression in bipotential immature cells, higher in parenchymal hepatobiliary cells and highest in BEC, which could be further subdivided into the lower expressing IHBD and higher EHBD (Yamasaki et al. 2006).

Higher expression in the capsular areas of the KO as well as portal areas shows the shared portal and capsular signalling mechanism has a HNF6 and HNF1β upregulating effect but only on CEBPα inhibited cells.

The EHBD is positive for JAG1 and NOTCH2 both throughout development and postnatally, beginning with high expression at E12.5. The liver parenchyma expresses low levels of both NOTCH2 and JAG1 from E12.5 onwards; by P0 its NOTCH2 expression was lost. JAG1 is expressed by endothelial and mesenchymal cells of the portal area by E12.5. From E15.5 onwards JAG1 mRNA was at high levels in the portal area. Some of the cells of the developing ducts in contact with the mesenchyme were JAG1+, while NOTCH2 was highly expressed throughout the developing ducts. At P0 IHBD still expressed JAG1 and NOTCH2 (Yamasaki et al. 2006). Expression in the portal area and developing duct suggest this signalling is BEC related. These expression patterns correlate with the levels of HNF6/HNF1β in the parenchyma, BEC and EHBD, suggesting a link between Notch signalling and the HNF6/HNF1β pathway.
JAG1 expression in the KO had the same pattern as that in the WT but at lower levels at E12.5, whereas NOTCH2 expression was higher in both the parenchyma and EHBD. At E15.5 pseudo ducts in the parenchyma had medium to low JAG1 and portal vein ECs had low levels. The parenchymal cells were heterogeneous for NOTCH2. At P0 all parenchymal cells were positive for JAG1 and it was higher in the portal areas. NOTCH2 in the parenchyma was higher than in the WT while the portal BECs were lower. So only NOTCH2 and not JAG1 correlates with the HNF6 expression levels of the KO (Yamasaki et al. 2006).

That the shared portalcapsular BEC differentiation signal may be notch based, due to the presence of JAG1 in portal areas and NOTCH2 in BECs and hepatoblasts, is countered by the absence of JAG1 in the capsule area. This suggests a different source of shared BEC inducing signal exists which may be the presence of shared ECM components between the two regions. (Yamasaki et al. 2006).

In the CEBPα KO HNF1β and HNF6 were upregulated in the pseudo duct structures of the liver showing that they are downstream of CEBPα. A direct affect is possible on HNF6 but is not demonstrated, suggesting CEBPα suppression in portal areas leads to increased HNF6 and then HNF1β expression. Notch and JAG1 are also upregulated in the pseudo duct structures in the non-portal areas compared to WT liver and so may also be downstream of CEBPα (Yamasaki et al. 2006). Notch signalling has been shown to downregulate CEBPα, HNF1α and HNF4α in hepatoblasts in vitro (Tanimizu and Miyajima 2004).

### 1.2.6 Hepatic Parenchyma development

The hepatic parenchyma is derived from the foregut endoderm. In vivo studies resulted in a model where TGFβ signalling controls the hepatic induction of endoderm by BMP and FGF signalling, restricting cells to an endoderm fate until the appropriate time (Wandzioch and Zaret 2009).

WNT signalling is required at different stages during hepatic development. WNT is repressed in the anterior endoderm, at E8.0, resulting in expression of HHEX and liver specification (McLin et al. 2007). Later in development, from E9.0, WNT acts in parallel with FGF and BMP to drive hepatogenesis (Ober et al. 2006, McLin et al. 2007, Goessling et al. 2008, Si-Tayeb et al. 2010). In zebrafish this WNT signalling comes from the lateral plate mesoderm adjacent to the endoderm. Studies of WNT2b mutants show it affects the timing of hepatic specification (Ober et al. 2006).
Initiation of hepatic development requires the FOXA proteins as shown in FOXA1/FOXA2 dual knockouts in vivo and in vitro (Lee et al. 2005). It may act as a pioneer factor for liver development changing the chromatin configuration of the genes necessary for liver specification (Kaestner 2005, Si-Tayeb et al. 2010).

HNF1β is necessary for the specification of the hepatic parenchyma. HNF1β KO mice form only the mesenchymal portion of the liver bud. The ventral endoderm of these mice fails to express albumin in response to FGF signalling (Lokmane et al. 2008). Hepatocyte specific fatty acid oxidation was also affected in the HNF1β LKO (Coffinier et al. 2002).

By E10.5 the invading endoderm cells have become CK+ hepatoblasts and grow further into the STM (Asahina et al. 2011). The hepatoblasts at this stage are bipotent with the ability to become hepatocytes or cholangiocytes (Si-Tayeb et al. 2010). HNF6 (OC-1) and OC-2 function redundantly and are necessary for this migration of the hepatoblasts into the STM during development (Margagliotti et al. 2007).

HNF4α and CEBPα are important regulators of hepatocyte differentiation and are highly expressed by the hepatoblasts which migrate into the septum transversum whereas HNF6 and HNF1β, involved in cholangiocyte differentiation, are expressed at low levels (Si-Tayeb et al. 2010). TBX3 KOs do not express HNF4α or CEBPα in the hepatoblasts but have increased levels of HNF6 and HNF1β (Lüdtke et al. 2009), showing that TBX3 promotes a hepatocyte over cholangiocyte fate. The inability of cells to delaminate in these KO models is suggested to be a consequence of a failed initiation of hepatocyte differentiation (Lüdtke et al. 2009, Si-Tayeb et al. 2010).

The transcription factors HNF1α, HNF1β, FOXA2, HNF4α, HNF6 and LRH-1 were found to interact with each other as well as other hepatic transcription factors to regulate maturation and terminal differentiation of hepatocytes (Kyrmizi et al. 2006).

HNF4α was found to be unnecessary for hepatic specification but required for further differentiation of the specified hepatic progenitors (Li et al. 2000, Parviz et al. 2003). HNF4α liver KOs, at the hepatoblast stage, results in a loss of endothelial cells and hepatocyte polarity, highlighting a role for it in cell junctions (Battle et al. 2006).

1.2.6.1 Hepatocyte zonation

The structural and functional heterogeneity of the liver is based upon its vasculature and the hepatocytes positions relative to it. For example catabolic processes occur in the portal vein area while anabolic ones occur in the central vein area (Coffinier et al. 2002). Expression of some genes of the hepatocytes is restricted relative to their proximity to
these portal and central zones (Jungermann and Katz 1989). The variation in expression can take the form of gradients with gradual changes in expression levels, or discrete areas with sudden changes (Spear et al. 2006).

WNT/β-catenin signalling has been shown to be involved in the zonation of hepatocytes (Kaestner 2009). In mice, the PC hepatocyte identity is β-catenin dependant and this effect is inhibited by APC in the periportal region (Benhamouche et al. 2006, Burke et al. 2009). HNF4α hepatocyte specific KOs have increased expression of PC specific genes (including GS) in the periportal hepatocytes. It was also shown that HNF4α directly binds the glutamine synthetase (GS) enhancer acting as a transcriptional repressor in periportal hepatocytes (Stanulovic et al. 2007). Activation of WNT was found to be sufficient to convert periportal gene expressing hepatocytes into PC ones. β-catenin activated the transcription factor LEF1 which in combination with HNF4α binds the promoters of PC genes and activates them, whereas HNF4α alone represses these genes. However in periportal hepatocytes portal specific genes were activated by binding of HNF4α to their promoter and there expression could be inhibited by LEF1 activation (Colletti et al. 2009).

GS and CPS1 are commonly used markers of hepatocyte zonation. These enzymes are involved in ammonia detoxification and glutamine homeostasis (Haussinger et al. 1992). Ammonia detoxification is carried out by the urea cycle, the first enzyme of which is CPS located in the periportal hepatocytes, while GS is restricted to PC ones. GS is also expressed at low levels in the developing intestine.

In mice all fetal hepatocytes are GS+, after birth GS expression in most hepatocytes decreases and it becomes restricted postnatally to the PC zone. 3-4 days postnatally expression is higher in a single layer of PC hepatocytes and after 12 days the adult pattern was established, a single layer of PC cells expressing GS. GS+ fetal and neonatal hepatocytes lack expression of other PC proteins found in the adult, suggesting independent establishment of zonation for different genes. Other PC antigens were not detected for 2-3 weeks (Bennett et al. 1987). GS mRNA in mice follows the same pattern. Total GS mRNA and transcription rate are similar at P1 and in adults, suggesting an increase in PC GS transcription and a decrease in the other cells (Kuo et al. 1988).

In adult humans CPS1 is concentrated in the portal area while GS is restricted to a 2-3 cell layer around the central vein. There is an intermediate zone in which neither enzyme is expressed. CPS1 is detectable from 5wpc in the human fetal liver. It is heterogeneously expressed until 9wpc when it becomes homogenous throughout the liver, until at least 2 days postnatally. GS is not detected in the human fetal liver. In 8 year human liver CPS expression and GS expression overlap with no intermediate zone which is clearly visible.
from 25 years onwards. Intensity and number of cells expressing GS decreased with age (Moorman et al. 1989).

The zonal expression of CPS and GS in human adults maps well to three zones of the liver acinus described by Rappaport (Rappaport 1973, Moorman et al. 1989). The absence of an intermediate zone between GS and CPS expression in adult rat may be explained by the apparent fixed size of these zones between species (Gaasbeek Janzen et al. 1987), meaning an intermediate zone is a feature of the larger human liver (Moorman et al. 1989).

GS was absent in humans up to 2 days after birth whereas in rodents GS is present from before birth (Lamers et al. 1987, Moorman et al. 1989). While CPS expression is found in 5wpc human (Moorman et al. 1989) but not at 13.5d rat (Gaasbeek Janzen et al. 1988), its equivalent (Butler H, Juurlink BHJ. An atlas for staging mammalian and chick embryos. CRC Press, Florida, 1987; 187-194.).

Different species have different developmental timing of birth, what is adult in one may appear at the fetal stage in another. Comparison of zonation development in the closely related rat and spiny mouse show that enzyme zonation is dependent on the development of the acinar structure and not on the timing of birth (Lamers et al. 1987). While perinatal adaptation causes an increase in expression of these enzymes, in whichever zone they are currently localised to (Lamers et al. 1987). This link between the initiation of zonation and the development of acinar structure is also shown to exist in humans where the fetal enzyme distribution is maintained in infants of 6 months and adult liver architecture is not found until 5 years of age. The authors hypothesised adult zonation patterns are initiated during this time though are still not complete in 8yr old liver as seen by the co-expression of CPS and GS in PC hepatocytes (Moorman et al. 1989).

In mouse GS expression is initially ubiquitous and then restricted to the central zone (Bennett et al. 1987). The rat also begins with both portal and central GS expression before restriction to the central zone. Human GS expression is postnatal. If human GS follows the same pattern as mouse and rat it will first be expressed ubiquitously before being restricted to its PC zone.

Different animals have different metabolic needs which may explain the variation in timing seen in the initial gene expression such as no human fetal GS. (Moorman et al. 1989).

In the rodent species studied PC restriction occurred before periportal restriction, this also fits with the human data with GS PC expression being overlapped by CPS expression in the 8 year old human liver. Showing central and portal zones are established independently of each other.
This variation between the onset of zonation and expression of functional enzymes in different rodent species and humans highlights the importance of human positive controls for comparison in stem cell derived models wherever possible and the potential shortcomings of relying on animal models of development. For example CPS-ve/GS-ve hepatocytes don’t exist in the adult rat but would be indicative of zone 2 hepatocytes in the adult human.

1.2.7 Laminin is associated with both fetal and portal phenotypes

Laminin is associated in the parenchymal cells with the induction of the early parenchymal and cholangiocyte markers as well as markers of continuous endothelium in the ECs. Up until 10wpc Laminin is found throughout the parenchyma as part of the sinusoidal ECM which maybe driving the early fetal phenotype of the parenchyma and preventing hepatocyte maturation. At this stage the LSECs express CD31/CD34 and have continuous cell junctions. Later in development the portal and peripheral areas are both laminin rich and induce duct formation in the CEBPα KOs (Yamasaki et al. 2006). In adult liver laminin marks the bile ducts, and is also in contact with the continuous endothelium of the PBP. In the Espanol-Suner paper inhibition of laminin production forced the injury induced hepatic progenitors into a BEC phenotype as opposed to a hepatocyte one (Espanol-Suner et al. 2012). It seems laminin is associated with the HNF1β/HNF6 expressing cells and continuous endothelium, first in development and later localised to the portal areas and also in the ECs during capillarization.

1.3 ESCs in liver differentiation

1.3.1 Differentiation over induction

Gene network analysis (Cellnet) revealed iHeps, fibroblasts converted to hepatocyte like cells by expression of HNF4α and FOXA1 capable of engrafting into Fah-/- mice and secreting albumin amongst other liver properties (Sekiya and Suzuki 2011), were in fact an induced endoderm progenitor also capable of long term colon engraftment (Morris et al. 2014). Using the same platform analysis of existing data sets showed cells derived by directed differentiation were more a like their in vivo targets, in their gene regulatory networks, than those derived by direct conversion. It was also apparent that directly converted cells still shared expression programs with their initial cell type. All of which
suggests that the most faithful derivation of an in vivo phenotype occurs through differentiation rather than direct conversion (Cahan et al. 2014).

The main difference found between differentiated and in vivo cells was a lack of promoter accessibility in the differentiated ones which was hypothesised to be due to a lack of induction of pioneer factors. This however was not the case in induced cells instead it was the expression of inappropriate gene regulatory networks from both the original cell population and aberrant cell types, resulting in hybrid cells (Cahan et al. 2014).

1.3.2 Examples of cholangiocyte differentiation and 3D culture

Studies of tubulogenesis in 3D culture show it occurs via a partial transient EMT (Yu et al. 2003). First the basolateral surface of a cell in the cyst extends and the cell proliferates resulting in a chain of cells which have lost epithelial polarity and resemble mesenchymal cells (Yu et al. 2003). Where cords of extended cells meet, lumens form and merge joining to the lumen of the original cyst and leaving a duct. This process concludes with the re-establishment of the polarized epithelial phenotype (Zegers et al. 2003).

1.3.2.1 HGF and EGF

In 3D gels of extracellular matrix epithelial cell lines can form cysts, which consist of monolayer spheres of polarised cells surrounding a central lumen. Addition of HGF to cells can induce or increase cyst formation and in some cases can induce branching morphogenesis and the formation of ducts (Brinkmann et al. 1995). Tanimizu et al (Tanimizu et al. 2007) investigated cyst formation in a liver progenitor cell line (HPPL) and found that both EGF and HGF can be used to increase the frequency of cyst formation and that efficiency was further increased by their use in combination. The efficiency of cyst formation was greatly reduced in the presence of a phosphatidylinositol 3-kinase (PI3K) inhibitor suggesting the PI3K pathway mediates the effect of EGF and HGF.

1.3.2.2 hES cell to cholangiocyte differentiation and characterisation

Zhao et al (Zhao et al. 2009) used activin A followed by BMP and FGF signalling to differentiate hES cells. They then used neural cadherin (N-cadherin) to select a hepatic endoderm population which expressed markers of hepatocyte and BEC lineages. When cultured on matrigel these progenitors were CK19+, CK7+ and AFP- like BEC cells. They were subsequently differentiated in a 3D culture system of matrigel and collagen I (COL1) in order to investigate the epithelial polarity of the cholangiocyte-like cells (CLC). These cells were CK19+, CK7+ and AFP- with β-catenin localised to the basolateral surface and
F-actin in the inner lumen, which is indicative of apico-basal polarity. They also formed round cysts with a central lumen. MDR, a transmembrane pump, has a secretory role in cholangiocytes (Gigliozzi et al. 2000). In the presence of an MDR inhibitor (verapamil), cholangiocyte-like cells could no longer transport fluorescent rhodamine 123 to a central lumen.
2 Stem cell-derived hepatocyte-like cells mimic fetal rather than adult hepatocytes by transcriptomic, proteomic and functional analyses

Melissa Baxter\textsuperscript{1*}, Sean Harrison\textsuperscript{1*}, Sarah Withey\textsuperscript{1*}, Rebecca Atkinson-Dell\textsuperscript{1}, Cliff Rowe\textsuperscript{1,2}, Dave T. Gerrard\textsuperscript{1,3}, Rowena Sison-Young\textsuperscript{2}, Roz Jenkins\textsuperscript{2}, Fang Zhang\textsuperscript{2}, Joanne Henry\textsuperscript{2}, Andrew Berry\textsuperscript{1}, Marie Best\textsuperscript{4}, Neil R. Kittingham\textsuperscript{2}, Chris E. Goldring\textsuperscript{2}, Karen Piper Hanley\textsuperscript{1}, Neil A. Hanley\textsuperscript{1,5}

\textsuperscript{1}Centre for Endocrinology & Diabetes, Institute of Human Development, Faculty of Medical & Human Sciences, University of Manchester, Manchester Academic Health Science Centre, AV Hill Building, Oxford Road, Manchester, UK

\textsuperscript{2}Department of Pharmacology & Therapeutics and MRC Centre for Drug Safety Science, University of Liverpool, Sherrington Building, Ashton Street, Liverpool, UK

\textsuperscript{3}Bioinformatics, Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester, UK

\textsuperscript{4}Human Genetics Division, University of Southampton, Southampton General Hospital, Tremona Road, Southampton, UK

\textsuperscript{5}Endocrinology Department, Central Manchester University Hospitals NHS Foundation Trust, Grafton St, Manchester, UK

* denotes equal contribution

Financial support

This work was funded by the Stem Cells for Safer Medicine Consortium (grants to NAH and CEG), the Engineering and Physical Sciences Research Council (EPSRC; to NAH), and a Medical Research Council (MRC) Centre grant. NH is a Wellcome Trust Senior Fellow (funded by WT088566 and WT097820). SW is a Biotechnology and Biological Sciences Research Council (BBSRC) PhD student.
2.1 Abstract

Hepatocyte-like cells (HLCs) differentiated from pluripotent stem cells offer utility for in vitro modelling of human liver function and toxicity. However, at present HLC maturity and whether any deficit represents a bona fide fetal state or aberrant differentiation have been unclear and compounded by comparison to adult hepatocytes with potentially deteriorated phenotype. To address these two issues, we implemented a differentiation protocol in multiple human embryonic stem cell (ESC) lines to produce HLCs comparable to those of others by increases in albumin secretion, alfa1-antitrypsin production, CYP3A activity and other transcript and protein parameters. Transcriptomic changes during differentiation mimicked human embryogenesis. Gene expression profile showed similarity to PC rather than periportal hepatocytes. Unbiased proteomics demonstrated greater proximity to liver than 30 other human organs or tissues. However, by specifically including fresh human fetal and adult hepatocyte controls, HLC maturity was shown by transcript, protein and function to be fetal-like and short of the adult phenotype. Expression of 51/63 phase 1 enzymes (81\%) was significantly upregulated in H9 HLCs and for 33 (52\%) was statistically no different from expression in fetal hepatocytes. HLCs secreted albumin and metabolized testosterone (CYP3A) and dextrophan (CYP2D6) comparably to fetal hepatocytes, which for the CYPs was \( \geq 100 \)-fold greater than HepG2 cells. Finally, in five tests devised by principal components analysis specifically to distinguish human hepatocyte maturity HLCs consistently demonstrated fetal not adult characteristics.

Conclusion: HLCs from different sources are broadly comparable with unbiased proteomic evidence for faithful differentiation down the liver lineage; however, at present, this phenotype mimics human fetal rather than adult hepatocytes.
2.2 Introduction

Hepatocyte-like cells (HLCs) differentiated from pluripotent stem cells (PSCs) offer promise as in vitro models of human liver development and function, hepatotoxicity and drug discovery (Baxter et al. 2010, Zhang et al. 2013). Most protocols have attempted mimicry of embryogenesis through the addition of soluble factors to the media. Activin-A (Agarwal et al. 2008, Hay et al. 2008, Basma et al. 2009, Song et al. 2009, Broelen et al. 2010, Roelandt et al. 2010, Si-Tayeb et al. 2010, Touboul et al. 2010, Cai et al. 2012, Medine et al. 2012, Hannan et al. 2013) either alone or with Wingless-related integration site (WNT) 3A (D'Amour et al. 2006, Hay et al. 2008, Roelandt et al. 2010, Chen et al. 2012, Medine et al. 2012) promotes definitive endoderm (DE)-like differentiation. Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) family members have been used for hepatic differentiation (Song et al. 2009, Broelen et al. 2010, Roelandt et al. 2010, Touboul et al. 2010, Cai et al. 2012, Medine et al. 2012, Hannan et al. 2013); and hepatocyte growth factor (HGF) and agents such as the synthetic glucocorticoid, dexamethasone (DEX), and oncostatin M (OSM) applied to generate cells of increased maturity (Agarwal et al. 2008, Hay et al. 2008, Basma et al. 2009, Song et al. 2009, Roelandt et al. 2010, Si-Tayeb et al. 2010, Touboul et al. 2010, Cai et al. 2012, Chen et al. 2012, Medine et al. 2012, Hannan et al. 2013). However, fully mature hepatocytes have not been produced, which raises two questions: are cells aberrant because human liver development has not been followed with adequate specificity; or, if the lineage is correct, how closely do HLCs mimic adult hepatocytes or are they ‘stuck’ in a fetal-like state? This is pertinent because of the widely reported detection of the ‘fetal marker’ alpha-fetoprotein (AFP) (Agarwal et al. 2008, Hay et al. 2008, Hay et al. 2008, Song et al. 2009, Broelen et al. 2010, Rashid et al. 2010, Roelandt et al. 2010, Si-Tayeb et al. 2010, Sullivan et al. 2010, Touboul et al. 2010, Chen et al. 2012, Hannan et al. 2013). Moreover, the maturity of HLCs is over-estimated if compared to sub-optimal human adult hepatocytes. Most commonly, hepatocyte controls have been cryopreserved for commercial availability and ease of shipping. Although thawed cells can match the metabolism of freshly isolated hepatocytes (Smith et al. 2012), comparison with HLCs has often required plated hepatocytes, which are more challenging to maintain (Hewitt et al. 2007). For instance, in a rare example where both cryopreserved and fresh cells were studied alongside HLCs, over 90% of the cytochrome P450 (CYP) 3A activity was lost in cryopreserved compared to freshly plated cells (Hay et al. 2011). This illustrates the risk of over-interpreting HLCs if compared solely against dedifferentiated hepatocyte controls. Furthermore, we have also
shown that human fetal hepatocytes possess proteins commonly interpreted as adult markers, such as CYP3A4 (Rowe et al. 2013).

The goal of this study was to address persisting questions about the differentiation and maturity of HLCs. We implemented a protocol with sufficient commonality and controls to allow comparison with multiple previous reports. We analysed a wide range of human ESC lines derived under different conditions alongside H9 cells, the most popular line for generating HLCs (Agarwal et al. 2008, Hay et al. 2008, Roelandt et al. 2010, Si-Tayeb et al. 2010, Touboul et al. 2010, Hay et al. 2011, Jozefczuk et al. 2011, Chen et al. 2012). HLCs were assessed by unbiased proteomics and a series of assays against fresh human fetal and adult hepatocytes. The analyses specifically included new tests systematically devised by unbiased proteomics and principal components analysis to distinguish between fetal and adult hepatocyte phenotypes (Rowe et al. 2013).
2.3 Materials and Methods

2.3.1 Human tissue and cells, and their culture

Human embryonic stem cell (ESC) lines were obtained with consent either directly from the derivation laboratory or the UK Stem Cell Bank. Cells were maintained on inactivated mouse embryonic fibroblast (MEF) cells (Baxter et al. 2009). The differentiation protocol (Supplemental Fig. 1) was commenced 3-4 days post-passage onto fresh MEFs using WNT3a (R&D Systems, UK) and Activin-A (Peprotech, UK), diluted in RPMI media (Sigma-Aldrich, UK); followed by BMP2, OSM, FGF2, HGF (all R&D Systems) and DEX (Sigma-Aldrich, UK), diluted in Hepatocyte Culture Medium (HCM) (Lonza, UK). Information on collecting the human fetal and adult hepatocyte controls, and sourcing and culturing HepG2 cells, has been reported previously (Rowe et al. 2013). In brief, fetal material was obtained from voluntary termination of pregnancy with informed consent under ethical approval. Samples ranged from 9 to 14 weeks post-conception (wpc). Fresh human adult hepatocytes were obtained and quality assessed as reported previously (Rowe et al. 2013). Hepatocytes were cultured in Williams’ E medium supplemented with 2mM L-Glutamine, Insulin-Transferrin-Selenium (ITS) and 100nM dexamethasone at 5% CO2 and 37°C by seeding cells onto Matrigel-coated 6-well plates overlaid 3h later with a second layer of Matrigel (ECM-sandwich) for analysis after 48h (Rowe et al. 2013).

2.3.2 Immunoblotting, immunofluorescence, cell proliferation and apoptosis studies

Immunoblotting and immunofluorescence were conducted as previously using antibodies shown in Supplemental Table 1 (Jennings et al. 2013, Rowe et al. 2013). Cell proliferation and apoptosis methodology is described in Supplemental Methods.

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<th>Dilution</th>
<th>Company</th>
<th>Fixative</th>
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<td>n/a (primary HRP conjugated antibody)</td>
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Supplemental Table 2-1 Details of antibodies in immunofluorescence and immunoblotting.

* Gift from R Juvonen, School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland.

2.3.3 Protein isolation and proteomic analysis

Protein isolation from whole cell extracts and labelling for isobaric tagging for relative and absolute quantification (iTRAQ) proteomics was described in Rowe et al (Rowe et al. 2013). Quantitation of proteins was relative to a common reference preparation included in each run across different experiments. Proteins identified by 2 or more peptides with
≥90% confidence, or by a single peptide with ≥99% confidence, were included in subsequent analyses. The dataset on whole cell extracts was filtered by statistical significance (P<0.05) using an unpaired t-test with Benjamini Hochberg adjustment. Proteins that were significantly upregulated >2-fold were interrogated against gene expression experiments deposited with the European Molecular Biology Lab / European Bioinformatics Institute (EMBL/EBI) Gene Expression Atlas (GEA; release 12.03.01; www.ebi.ac.uk/gxa/) (Kapushesky et al. 2012) using the filter of up or down-regulated in at least one experiment on the GEA database.

2.3.4 Biochemical assays
The secretion of albumin and urea into the media was measured using a human albumin enzyme-linked immunosorbent assay (ELISA) kit and QuantiChrom™ urea assay kit (both from Bethyl Laboratories). Comparison to secretion from fetal and adult hepatocytes was assessed by unpaired two-tailed Student’s t-test. CYP3A activity was assessed in duplicate by incubation with P450-Glo™ CYP3A4 Assay reagent (Luciferin-PFBE; Promega Ltd). For CYP analysis by mass spectrometry cells were incubated with 1mM testosterone or 1mM dextromethorphan (Sigma, UK) in HCM. Conditioned medium was collected and diluted 1:1 in 0.5µM phenacetin (Sigma) stop solution in methanol. CYP activity was calculated per min incubation. Alcohol dehydrogenase activity of cell lysates was assessed using a detection kit following manufacturer’s instructions (Abcam, UK). Results were standardized to amount of protein measured by Bradford assay.

2.4 Supplementary Methods
2.4.1 Cell proliferation and apoptosis studies
Bromodeoxyuridine (BrdU; 10µM; Sigma) was incubated with cells at the end of Stage 2 for 4h at 37°C. Cells were washed with phosphate buffered saline (PBS), dissociated with TrypLE Express (Invitrogen), centrifuged, resuspended in 200µl of ice-cold PBS / EDTA (5mM) and fixed in ice-cold ethanol. Nuclei were released with pepsin (0.4mg/ml in 0.1N HCl; Sigma) for 30min at room temperature followed by washing in PBS, re-suspension in 2N HCl, incubation in the dark for 30min at 37°C, prior to treatment with 0.1M sodium tetraborate. Further washing in PBS was followed by treatment for 30min with RNaseA (10µg/ml; R4875 Sigma). Nuclei were washed, resuspended and incubated in 10µl containing anti-BrdU antibody (AlexaFlour®488 conjugate, Millipore,) or IgG₂a control
(AlexaFlour® 488 conjugate, Santa Cruz) at 4°C overnight. All nuclei were stained with propidium iodide (50µg/ml; Sigma) and analysed by flow cytometry within 48h. Apoptosis was studied in dissociated hepatoblast-like cells with the NucView 488 caspase 3 kit (Biotium Inc) according to the manufacturer’s instructions prior to analysis by flow cytometry. In both cell proliferation and apoptosis studies 10,000 cells were gated with aggregates and debris excluded from analyses.

**2.4.2 RNA-sequencing and quantitative RT-PCR analysis of genes encoding phase 1 enzymes**

RNA sequencing (RNA-seq) libraries were generated using the TruSeq® Stranded mRNA assay (Illumina, Inc.) according to the manufacturer’s protocol. Briefly, total RNA (1µg) was used as input material from which polyadenylated mRNA was purified using polyT, oligo-attached, magnetic beads. mRNA was reverse transcribed into first strand cDNA using random primers. Second strand cDNA was synthesized using DNA Polymerase I. Following a single ‘A’ base addition, adapters were ligated to the cDNA fragments, and the products then purified and enriched by PCR to create the final cDNA library. The loaded flow-cell was then paired-end sequenced on an Illumina HiSeq2000. Demultiplexing of the output data (allowing one mismatch) and BCL-to-Fastq conversion was performed with CASAVA 1.8.3. Paired end reads were mapped to hg19 using TopHat (version 1.4.1) (Trapnell and Salzberg 2009) and visualized using the UCSC Genome Browser (http://genome.ucsc.edu/) (Kent et al. 2002). Gene-level transcription abundances (read counts) were calculated in the Partek Genomics Suite (version 6.6 (6.12.1227); Partek Inc., St. Louis, MO, USA). Following mapping, read counts were filtered for mitochondrial genes, ribosomal RNAs and two other multilocality RNAs ("Metazoa_SRP", “7SK”).

Phase 1 enzyme expression was analysed in triplicate by reverse transcription and quantitative PCR using Human Phase 1 Enzymes PCR Array and accompanying reagents according to the manufacturer’s protocols (SABiosciences Ltd). Total RNA was extracted and genomic DNA eliminated for each sample. A reverse transcription cocktail mix was prepared as instructed and was added to a standardized amount of each RNA sample. First strand cDNA synthesis was carried out at 42°C for 15min. A bulk PCR reaction mix was made consisting of RT² SYBR® Green qPCR Mastermix, H₂O and template cDNA, aliquots of which were then added to each well of the array plate. PCR was performed on an ABI Step-one instrument using a two-step Cycling Program of 95°C for 10min and 40 cycles of 95°C (15sec) and 60°C (60sec). Fold changes in expression of HLCs and fetal and adult hepatocytes over undifferentiated cells were calculated by the ΔΔC_t method.
standardized against the combined expression of four housekeepers (*HPRT1, RPL13A, GAPDH* and *ACTB*) (*B2M* was unreliable in our experience as a housekeeping control). Data were analysed using the RT² PCR Array Data software (SABiosciences). *P*-values were calculated for comparing groups based on a two-tailed Student’s t-test of the triplicate data for each gene.
2.5 Results

2.5.1 Differentiation of human ESCs towards HLCs

Based on previous studies (D’Amour et al. 2006, Agarwal et al. 2008, Hay et al. 2008, Hay et al. 2008, Basma et al. 2009, Song et al. 2009, Brolen et al. 2010, Roelandt et al. 2010, Si-Tayeb et al. 2010, Touboul et al. 2010, Cai et al. 2012, Chen et al. 2012, Medine et al. 2012, Hannan et al. 2013) and iteration we developed a 3-stage protocol (Supplemental Fig. 1) to differentiate a range of human ESC lines derived under diverse conditions to HLCs. During Stage 1 to definitive endoderm (DE)-like cells Brachyury protein was increased by Activin-A on the second and third days, at and after which FOXA2, GATA4 and SOX17 increased (Fig. 1A). However, the low serum (0.5% FBS) caused significant cell death. This was prevented by including WNT3A (25ng/ml) for the first two days of culture (D’Amour et al. 2006, Hay et al. 2008, Roelandt et al. 2010, Chen et al. 2012, Medine et al. 2012) leading to robust detection of the three nuclear transcription factors by day 4 (Fig. 1B). FOXA2, SOX17 and GATA4 were detected in >50% of cells for each ESC line indicating a shared but variable propensity for DE-like differentiation. MAN1, SHEF1 and HUES8 converted less efficiently while more homogeneous differentiation was observed in H9 cells (77-98% of cells positive for FOXA2, SOX17 and GATA4) and HUES7 cells (84-96% for the three transcription factors) (Fig. 1C and Supplemental Fig. 2).
SUPPLEMENTAL FIG. 1: Baxter et al

**STAGE 1a / Definitive endoderm-like: 2 days (→ DAY 2)**
RPMI + 1mM L-glutamine (L-G), 0.5% FBS, Activin-A (Act-A) (100ng/ml), Wnt3a (25ng/ml)

**STAGE 1b / Definitive endoderm-like: 2 days (→ DAY 4)**
RPMI + 1mM L-G, 0.5% FBS + Act-A (100ng/ml)

**STAGE 2 / Hepatoblast-like: 6 days (→ DAY 10)**
Hepatocyte culture medium (HCM), Bmp2 (20ng/ml) + Fgf4 (30ng/ml)

**STAGE 3a / Hepatocyte-like: 5 days (→ DAY 15)**
HCM + Hgf (20ng/ml)

**STAGE 3b / Hepatocyte-like: 15 days (→ DAY 30)**
HCM + Osm (10μg/ml), Dexamethasone (100nM)

Supplemental Figure 2-1 The three-stage differentiation protocol.

FIGURE 1: Baxter et al

A Day 0 1 2 3 4
Brachyury FOXA2 GATA4 SOX17 β-Actin

B FOXA2 SOX17 GATA4 β-Actin

C FOXA2 SOX17 GATA4
Figure 2-1 Stage 1 differentiation of ESCs to definitive endoderm-like cells.

A. Immunoblotting of ESCs differentiated for 4 days towards definitive endoderm (DE) by the addition of Activin-A. In the absence of WNT3A, transient Brachyury was observed but cell death limited the subsequent detection of FOXA2, GATA4 and SOX17 at Day 4. B. Inclusion of WNT3A for Stage 1a (see Supplemental Fig. 1 for the protocol) led to robust detection of FOXA2, SOX17 and GATA4 by immunoblotting and immunofluorescence from DE-like cells at the end of Day 4. Size bar represents 25µm. C. Mean percentage of cells by count (+/- S.E; numbers represented by the bar are shown below) that contained nuclear FOXA2, SOX17 or GATA4 either alone or in combination with NANOG at the end of Stage 1. Of the five different ESC lines H9 and HUES7 showed the best conversion to DE-like cells. Both immunoblotting and immunofluorescence were carried out on 3 independent experiments.

Supplemental Figure 2-2 Immunofluorescence for the differentiation of all five human ESC lines to definitive endoderm-like cells.

Immunofluorescence data indicative of those that are summarized graphically in Figure 1C. Size bars represent 150µm (A) and 50µm (B).

At the end of Stage 2 (hepatoblast-like cells), 91% of HUES7- and 98% of H9-derivatives contained cytoplasmic AFP of which at least two-thirds clearly dual-stained for nuclear HNF4α (Fig. 2A-B). Approximately 25% of these AFP+ cells were proliferating according to BrdU uptake over 4h, with 10-15% in apoptosis as measured by Caspase-3 activity for both HUES7 and H9 derivatives (Fig. 2C).
Figure 2-2 Stage 2 differentiation to hepatoblast-like cells.

A-B. Immunofluorescence for HNF4α and AFP and their quantification by cell counting in HUES7 and H9 cells at the end of Stage 2 (the example in panel A is for H9 cells). The quantification of HNF4α-positive cells only included robustly stained nuclei and therefore is most likely an underestimation of the entire population of dual-stained HNF4α+/AFP+ cells. Size bar represents 25μm. The pie charts show the averages of at least three separate differentiation experiments. C. Proliferation and apoptosis in the HNF4α+/AFP+ cell population for the HUES7 and H9 lineages. Proliferation was assessed following BrdU incorporation over 4h. Apoptosis was
assessed by caspase 3 activity (see Methods). The data shown are the mean +/- S.E. of three separate differentiation experiments.

### 2.5.2 Initial characterization of HLCs

Transcripts for *albumin* and *alpha1-antitrypsin* (*AAT*, officially designated *SERPINA1*) were barely identified by RNA-seq of DE-like cells (end of Stage 1) but were readily detected in early HLCs following Stage 3A (Fig. 3A-B). *AAT* transcripts were largely initiated from the first coding exon as in human embryonic and adult liver rather than annotated upstream exons (Fig. 3B and data not shown). We have shown that the transcription factors, GATA4 and SOX17, become restricted from the early human embryonic liver compared to the adjacent foregut (Jennings et al. 2013). Between DE-like cells and early HLCs, GATA4 and SOX17 expression declined by approximately 75% and >90% respectively (data not shown). The transcription factor HNF4α, encoded by *HNF4A*, is a master regulator of the hepatocyte phenotype (Parviz et al. 2003, Hwang-Verslues and Sladek 2010, DeLaForest et al. 2011). During liver development in vivo there is a switch from an upstream ‘fetal/immature’ P2 promoter to a downstream ‘adult/mature’ P1 promoter generating alternative first exons of the *HNF4A* gene (Nyirenda et al. 2006, Harries et al. 2008). This was mirrored in our cultures: in DE-like cells, *HNF4A* was expressed from the ‘immature’ P2 promoter; however, in HLCs the downstream first exon was preferentially transcribed from the P1 ‘adult/mature’ promoter (Fig. 3C, red boxes).
Figure 2-3 Differentiation to early hepatocyte-like cells (Stage 3a).

A-C. RNA-seq read counts for albumin, alpha1-antitrypsin (SERPINA1) and HNF4A displayed on the y-axes and annotated using the UCSC Human Genome Browser for HUES7 HLCs. A-B. Albumin and AAT expression was barely detected in DE-like cells but readily apparent at the end of Stage 3a. Red boxes in B show the transcription start site (TSS) at the first coding exon for AAT and negligible reads from upstream exons (box to the right). C. Red boxes show the shift in usage from the P2 to the P1 promoter for HNF4A between DE-like cells and HLCs. Data represents 2 independent samples from each stage.

Whether HLCs mimic periportal and PC hepatocytes has not been examined previously. Glutamine synthase, a PC marker, was readily detected by immunocytochemistry in contrast to carbamoyl-phosphate synthase, a periportal protein (Supplemental Fig. 3A). This correlated to their transcript profiles assessed by RNA-seq (Supplemental Fig. 3B) and was similarly true for a range of other genes reported as differentially expressed between PC and periportal hepatocytes (Stanulovic et al. 2007). All the PC genes except UDP-glucuronosyltransferase 1A were expressed at increased or equivalent levels to those...
in DE-like cells. In contrast, only phosphoenolpyruvate carboxykinase 2 of the periportal markers was expressed in HLCs, but at levels lower than in DE-like cells. Taken together, these data indicate an HLC phenotype more compatible with PC than periportal hepatocytes.

Supplemental Figure 2-3 Hepatocyte-like cells show PC rather than periportal features.

A. Immunofluorescence on HUES7 HLCs showing detection of the PC hepatocyte marker, glutamine synthase, but not the periportal marker, carbamoyl-phosphate synthase 1. B. Accompanying transcript analysis by RNA-seq for glutamine synthase and carbamoyl-phosphate synthase 1 showing read counts on the same scale on the y-axis in both DE-like cells and HLCs. Further tracks are shown for other PC- and periportal-restricted transcripts (Stanulovic et al. 2007). Expression in HLCs of glutamine synthase, ornithine aminotransferase, apolipoprotein E, UDP-glucuronosyltransferase 1A and thyroid hormone receptor β, all indicative of PC hepatocytes (Stanulovic et al. 2007), is greater than or equivalent to levels in DE-
like cells. In contrast, periportal markers, *carbamoyl phosphate synthase 1*, *ornithine carbamoyltransferase* and the catalytic subunit of *glucose-6-phosphatase*, were not detected in DE-like cells or HLCs. *Phosphoenolpyruvate carboxykinase 2* was expressed in HLCs but at lower levels than those detected in DE-like cells. Data represents 2 independent samples from each stage.

Final HLC morphology mimicked that of freshly plated human adult hepatocytes (Fig. 4A). Across the five different ESC lines, albumin was present in >75% of differentiated H9, SHEF1 and HUES7 cells (Fig. 4B). At least half of the H9 and HUES7 albumin-positive HLCs also contained AAT (Fig. 4B-C). All five cell lines showed progressive increase in albumin secretion during Stage 3 of differentiation (from Day 11; Fig. 4D). At the end of differentiation, levels were comparable or superior to those from freshly plated fetal hepatocytes, which were approximately 8-fold lower than from freshly plated adult hepatocytes. HLCs also showed urea secretion (mean +/- S.E.: 2.87 +/- 0.18 μg/ml/mg protein/day) comparable to fresh fetal hepatocytes (2.71 +/- 0.09 μg/ml/mg protein/day), but approximately 19-fold lower than freshly plated adult cells (Fig. 4E).
Figure 2-4 Characterization of hepatocyte-like cells at the end of Stage 3.
A. Brightfield image of HLCs in comparison to freshly plated human adult hepatocytes (example shown is for H9). Size bar represents 150μm. B-C. Quantification by cell counting (mean +/- S.E. from 3 experiments) of dual immunofluorescence for albumin and α1-antitrypsin (AAT) in the five human ESC lines counterstained with DAPI. C. Immunofluorescence shown as an example for H9 cells. Size bar represents 50μM. D. Albumin secretion and (E) Urea secretion into the media (mean +/- S.E. from at least three experiments) for the five human ESC lines at sequential time points during differentiation compared to secretion from equivalent numbers of freshly plated human fetal and adult hepatocytes. NS, no significant difference between day 23 and day 30 values for all lines (except HUES7 at day 30, *p<0.05) compared to fetal samples. Values for all lines except HUES8 (NS) were significantly lower (p<0.05) than adult samples.

2.5.3 The HLC proteome mimics liver more than other organ or tissue types

While these data were encouraging of liver-specific differentiation, as with previous analyses of HLCs, the assumption is based on a limited number of user-selected proteins and assays, and ignores potentially greater similarity to cell-types from other organs. We performed unbiased assessment by iTRAQ proteomics of whole cell extracts from undifferentiated H9 cells and the corresponding HLCs at the end of Stage 3. Sixty-one proteins showed statistically significant upregulation (>2-fold) including known liver markers, such as AAT/SERPINA1, various cytokeratins, and phase 1 (e.g. aldehyde dehydrogenases) and phase 2 enzymes [e.g. nicotinamide N-methyltransferase (NNMT) and glutathione S-transferase (GST) Mu3] (Supplementary Table 2). This collection of proteins was compared against data from 30 other human organs and tissue types in the EBI Gene Expression Atlas (Fig. 5). By heatmap, the upregulated HLC proteome most closely resembled gene expression in liver followed by another anterior derivative of foregut endoderm, the thyroid. Other foregut endoderm derivatives (stomach, small intestine and pancreas) showed recognizable similarity, in contrast to marked divergence of HLCs from mesodermal and ectodermal derivatives of bone marrow, skeletal muscle and brain. Thus, the proteome of our HLCs resembled that of liver cells more than cell-types from any other human organ or tissue tested.

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**Supplemental Table 2-2 Proteins upregulated in H9 hepatocyte-like cells.**

The table shows the list of proteins where the mean triplicate value was increased more than 2-fold and statistically significant compared to undifferentiated cells.
Figure 2-5 Proteins upregulated in HLCs characterize liver more than other human organs and tissues.

A heatmap of the 61 proteins significantly upregulated >2-fold in H9 HLCs compared to undifferentiated ESCs (Supplemental Table 2) analysed against gene expression experiments from a wide range of human organs and tissues deposited in the EMBL/EBI Gene Expression Atlas (GEA). The numbers in individual red or blue
boxes represent the number of experiments deposited in the GEA database where the gene encoding that particular gene was up- (red) or down-regulated (blue) in the relevant organ or tissue type.

2.5.4 HLCs have a metabolic profile comparable to human fetal rather than adult hepatocytes

Because we wanted to gain broad developmental insights into the HLC phenotype the iTRAQ analyses were performed on whole cell extracts rather than enriched microsomes. As a consequence, this restricted identification of phase 1 enzymes, especially CYPs, identical to our previous study with human fetal and adult hepatocytes (Rowe et al. 2013). Therefore, we used quantitative RT-PCR to analyse the expression of phase 1 enzymes, including CYPs (Fig. 6A), alcohol dehydrogenases (Fig. 6B), flavin-containing monoxygenases (Fig. 6C), aldehyde dehydrogenases (Fig. 6D), esterases (Fig. 6E) and other enzymes (Fig. 6F). Of the 84 genes analysed using commercial plates, 21 were excluded as inadequate either because detection in the fresh human adult hepatocytes was very low or because expression was more characteristic of other organs (e.g. CYP11B2 catalyzes the production of cortisol in the adrenal cortex). This left 63 enzymes by which to judge HLCs; the expression of 51 (81%) was significantly increased in H9 HLCs and 30 (48%) were significantly increased in HUES7 HLCs compared to their undifferentiated counterparts. CYP3A family members, CYP1B1, and DPYD were clearly increased in HLCs (Fig. 6A) with more modest increases in a number of alcohol dehydrogenases and flavin-containing monoxygenases (Fig. 6B-C). The increase in the aldehyde dehydrogenase gene, ALDH1A1, in H9 HLCs approached levels in fetal hepatocytes while ALDH1A2 levels surpassed those of both adult and fetal cells (Fig. 6D). However, HLC transcript levels were commonly markedly reduced compared to those in fresh human adult hepatocytes. In contrast, expression of 33 of the 63 (52%) phase 1 enzymes in H9 HLCs was statistically either greater or no different than in the fresh human fetal hepatocytes indicating a major overlap between the HLC and fetal phase 1 metabolic phenotype.
Figure 2-6 Quantitative RT-PCR expression analysis of genes encoding phase 1 enzymes.
Gene expression in HLCs quantified as fold difference over levels in the corresponding undifferentiated ESCs. Fold difference in fresh human adult hepatocytes (orange numbers above the bars) and fresh human fetal hepatocytes (pink numbers above the bars) are relative to levels in undifferentiated H9 cells. Genes shown encode: A. cytochrome P450 (CYP) enzymes; B. alcohol dehydrogenases; C. flavin-containing monooxygenases; D. aldehyde dehydrogenases; E. other enzymes; and F. esterases. Red (H9 HLCs) and green (HUES7 HLCs), orange (fetal) or pink (adult) numbers. All showing the mean from 3 independent experiments.

CYP3A activity, mostly via CYP3A4 in adult liver, and CYP2D6 are two major mechanisms for drug metabolism that require CYP oxidoreductase (CYPOR) to donate electrons during catalysis. We have shown that CYP3A and CYP2D6 immunoreactivity can be detected in human fetal hepatocytes (Rowe et al. 2013). To help further gauge maturity of our HLCs, we measured protein levels by immunoblotting and metabolic activity either by commercial luciferase assay or mass spectrometry. Immunoreactivity for CYP3A (current antibodies fail to distinguish the different CYP3A isoforms) in HLCs was approximately 10% of levels in fresh adult hepatocytes (Fig. 7A). For CYP2D6, two closely positioned bands were visible, the upper of which corresponded to the size of purified protein (Fig. 7A). CYPOR was robustly detected. CYP2D6 metabolizes dextromethorphan to dextrorphan. This activity was detected in both H9 and HUES7 HLCs, but not in ESCs or HepG2 cells. Conversion by HUES7 HLCs was similar to that by fetal hepatocytes but was 527-fold less than detected in fresh adult hepatocytes (H9 HLCs values were 1313-fold lower than for adult cells) (Fig. 7B). CYP3A-mediated metabolism of testosterone to 6β-hydroxytestosterone by HLCs was at least 100-fold greater than by ESCs or HepG2 cells and at least comparable to fresh fetal hepatocytes (Fig. 7C). Nevertheless, CYP3A metabolism of testosterone was 47-fold and 66-fold higher in fresh adult hepatocytes than in HUES7 and H9 HLCs respectively. Mass spectrometry provides a ‘gold standard’ for metabolic assay. In contrast, our experience of measuring CYP3A activity by commercially available luciferase assay (PFBE reagent, Promega) was unhelpful. Although HLCs from all five ESC lines matched fresh fetal hepatocytes, fetal hepatocytes actually showed greater activity than their adult counterparts (Fig. 7D).
Figure 2-7 HLCs match fetal hepatocytes for CYP3A and CYP2D6 activity.

A. Immunoblotting for CYP3A, CYP2D6 and CYPOR in HLCs. Equivalent numbers of human adult hepatocytes contained approximately 30 fmol of CYP3A and 20 fmol of CYP2D6. B. CYP2D6 metabolism of dextromethorphan to dextrorphan. Conversion in fresh adult hepatocytes was 200 nmol/min/million cells. C. CYP3A metabolism of testosterone to 6β-hydroxytestosterone. Conversion in fresh adult hepatocytes was 3,300 nmol/min/million cells. D. CYP3A luciferin assay. Bar graphs show mean +/- S.E. from at least three independent experiments.
2.5.5 Specific tests to distinguish hepatocyte maturity show that HLCs are fetal-like

We have previously shown that CYP3A4 protein detected by iTRAQ is relatively ineffective at determining hepatocyte maturity or whether cells have dedifferentiated (Rowe et al. 2013). In contrast, principal components analysis provided new protein combinations and simple assays not requiring mass spectrometry to distinguish human adult hepatocytes from their fetal counterparts: AFP, glutathione S transferase π (GSTp) and heat shock protein (HSP) 47, with negligible alcohol dehydrogenase (ADH) activity and CYP2A6, discriminate fetal cells; conversely, abundant CYP2A6 and ADH activity are hallmarks of adult cells (Rowe et al. 2013). AFP, GSTp, and HSP47 were readily detected in our HLCs (Fig. 8). CYP2A6 could be weakly detected in HLCs, which also showed slightly more ADH activity than fetal cells; however, both CYP2A6 levels and ADH activity were much higher in adult hepatocytes. These data strongly indicate a fetal phenotype for the HLCs.
Figure 2-8 HLCs are fetal-like based on tests specifically designed to distinguish hepatocyte maturity.

Specific investigations determined by principal components analysis can distinguish human fetal from adult hepatocytes (Rowe et al. 2013). A. Immunoblotting of four independent preparations of HLCs for the fetal marker AFP. B. Immunoblotting of fresh adult (lanes 1 and 2) and fetal hepatocytes (lanes 3 and 4) each from separate donors and two independent HLC preparations (lanes 5 and 6) for the most discriminatory adult hepatocyte marker CYP2A6 and fetal markers, GSTp and HSP47 (Rowe et al. 2013) with β-actin control. C. Assay for alcohol dehydrogenase activity in HLCs compared to fetal and adult hepatocytes. Mean +/- S.E. from three experiments using different HLC preparations and different fetal and adult specimens.

2.6 Discussion

The same soluble factors have been common to most HLC differentiation protocols. Our differentiation of DE-like cells matched others in its sequential use of Activin-A (Agarwal et al. 2008, Hay et al. 2008, Basma et al. 2009, Song et al. 2009, Brolen et al. 2010,

Four features of our data extend confidence that our protocol, and thus closely related ones (D’Amour et al. 2006, Agarwal et al. 2008, Hay et al. 2008, Hay et al. 2008, Basma et al. 2009, Song et al. 2009, Brolen et al. 2010, Roelandt et al. 2010, Si-Tayeb et al. 2010, Touboul et al. 2010, Cai et al. 2012, Chen et al. 2012, Medine et al. 2012, Hannan et al. 2013), mimic normal human liver development. Firstly, the down-regulation of SOX17 and GATA4 expression between DE-like cells and HLCs [here and (Hay et al. 2008, Si-Tayeb et al. 2010, Touboul et al. 2010)] complies with the exclusion of these transcription factors that we showed in human liver bud as it develops from foregut between 4 and 5 wpc (Jennings et al. 2013). The high rates of proliferation and apoptosis during this phase of differentiation match those seen previously (DeLaForest et al. 2011) and are consistent with the major remodelling that takes places during embryogenesis. Thirdly, we mimicked the change in HNF4A promoter usage during hepatocyte maturation (Nyirenda et al. 2006, Harries et al. 2008). This occurred prior to adding dexamethasone, presumably due to other adrenocortical steroids in the media. Finally, we demonstrated that the HLC proteome had more in common with human liver than any other organ tested. Unfortunately, comparison to our published human fetal and adult hepatocyte datasets was precluded by exhaustion of the original common reference pool (Rowe et al. 2013). Nevertheless, the unbiased protein data complement a previous ‘liver-specific’ transcriptome signature defined in HLCs (Si-Tayeb et al. 2010). The latter emanated from microarray analysis of multiple adult human tissues (Ge et al. 2005). ‘Liver-specific’ genes, such as albumin, various CYPs, and AFP,
can be expressed more widely than in liver (Baxter et al. 2010). Hence, reassurance comes from our proteomic approximation of HLCs to liver with some similarity to related endoderm fates but marked disparity from major ectoderm and mesoderm lineages. Uncertainty on the extent of HLC maturity has been discussed by others (Hay et al. 2008, Jozefczuk et al. 2011), including the lack of functional comparison with human fetal hepatocytes (Hannan et al. 2013). In addition, while cryopreserved adult hepatocytes can perform well in short term incubations in suspension (Hewitt et al. 2007, Smith et al. 2012) plating can lead to a deteriorated phenotype compared with fresh cells (Hay et al. 2011). We included undifferentiated stem cells and both fresh first trimester fetal and adult hepatocytes and consistently demonstrated major similarities with the fetal rather than the adult cell-type. Moreover, by studying fold increments over undifferentiated stem cells our data can be integrated with those from others. Our fetal-like levels of albumin secretion from HLCs showed at least 200-fold increase from the parent ESC line (Fig. 4D). This increment matches or surpasses that reported by others (Agarwal et al. 2008, Song et al. 2009, Rashid et al. 2010, Roelandt et al. 2010, Hay et al. 2011, Zhang et al. 2011, Woo et al. 2012) but was still lower on average than secretion from freshly plated adult cells, also seen by others (Song et al. 2009, Hay et al. 2011). However, lower increments in albumin secretion from ESCs to HLCs have been reported to match adult hepatocytes following cryopreservation (Zhang et al. 2011, Woo et al. 2012). These data imply the ease with which adult control cells can dedifferentiate and that in fact HLCs are much more similar to first trimester fetal cells in keeping with the widespread detection of AFP (Agarwal et al. 2008, Hay et al. 2008, Hay et al. 2008, Song et al. 2009, Brolen et al. 2010, Rashid et al. 2010, Roelandt et al. 2010, Si-Tayeb et al. 2010, Sullivan et al. 2010, Touboul et al. 2010, Chen et al. 2012, Hannan et al. 2013). A similar fetal-like conclusion can be drawn from urea secretion. Others have observed relatively low urea secretion in HLCs compared to adult cells (Song et al. 2009, Roelandt et al. 2010, Zhang et al. 2011), which may also reflect a PC rather than periportal phenotype. Urea synthesis is a feature of periportal hepatocytes (Stanulovic et al. 2007). During development periportal cells emanate from the duct plate and migrate centrally (Furuyama et al. 2010, Carpentier et al. 2011) while at least some PC cells may differentiate via a different route, in keeping with the presence of human embryonic liver well before the ductal plate at approximately 8 wpc (Jennings et al. 2013, Rowe et al. 2013). Our HLCs possessed multiple markers of PC but not periportal cells. This raises the possibility that new protocols may be required to mimic the development of hepatic ductal plate from which periportal HLCs and bile ducts differentiate.
H9 HLCs matched fetal hepatocytes for the expression of half of the CYPs tested but were inferior for others (e.g. CYP2C8, CYP3A, CYP4 and CYP7 family members, and CYP8B1). This included widespread lower level transcripts for what have been previously considered ‘adult’ CYPs (Hannan et al. 2013) warning that detection alone is not a reliable indicator of maturity. Phase 1 enzyme expression was commonly massively higher for fresh adult cells (up to 62,000-fold over ESCs for CYP2E1). This was not apparent in a recent protocol (Shan et al. 2013) where assessment of differentiation included cryopreserved adult hepatocytes that had transcript levels only approximately 20-fold higher than undifferentiated stem cells for CYP2B6 (445-fold here), 10-fold for FMO3 (8,178-fold here) and 20-fold for CYP2A13 (664-fold here). In fact, the human fetal liver contains both CYP3A4 and CYP2D6 proteins prior to mid-gestation (Rowe et al. 2013). Here we showed fetal-like HLC function by mass spectrometry despite HLCs possessing between 10- and 60-fold lower transcript levels than fetal hepatocytes for CYP3A4, CYP3A5 and CYP3A7 (Fig. 6). Our experience with a commonly used commercial ‘CYP3A4’ luciferase assay was misleading. Luciferase CYP3A activity from HLCs was equivalent to data from others (Chen et al. 2012) and to fetal hepatocytes, but fetal hepatocytes actually showed more luciferase activity than adult hepatocytes, which is inconsistent with the approximate 100-fold superiority of adult cells by mass spectrometry.

In summary, our data show that HLC differentiation mimics human development and extend confidence that our HLCs and those of others are genuinely and specifically part of the hepatocyte lineage. However, by a range of analyses, these HLCs share pronounced similarities with human fetal rather than adult hepatocytes, implying currently elusive factors require discovery to transition HLCs in vitro to a truly mature adult phenotype.

### 2.7 Acknowledgements

The authors thank Cellins Vinod, Leena Abi and staff at St Mary's Hospital for assistance in collecting samples and Risto Juvonen from the University of Eastern Finland, Finland for gifting anti-CYP2A6 antibody.
3 Human ESC differentiation to hepatic organoids

3.1 Abstract

While the use of PSC derived hepatocytes in disease and toxicity has been explored, these models lack the complex interactions between multiple cell types which occurs in vivo. We address this by directed differentiation of human ESCs to hepatic organoids which contain multiple cell types in a 3D culture system. These organoids contain polarised cells which share markers, morphology and structure of the liver including; HNF4α+ HLCs surrounding multidrug resistance protein 2 (MRP2) positive canaliculi-like channels, CK7+ CLCs organised into duct structures with a central lumen, LYVE1+/CD34+ branching structures with signs of endothelial polarity and associated pericytes, mesenchymal cells positive for markers of HSCs and an outer layer of cells positive for mesothelial markers and ECM proteins. Comparison with primary human tissue shows these cells more closely resemble fetal rather than adult liver, as found previously. Treatment with the cholangiotoxin DAPM reveals a complex dose response involving the CLCs and HLCs which matches previously described in vivo studies, including the secretion of hepatic enzymes and proteins as well as signs of regeneration.
3.2 Introduction

Current liver targets for PSC derived cell therapy involve the generation of HLC with specific loss of function such as metabolic disorders, which are then used to model (Ghodsizadeh et al. 2010, Rashid et al. 2010, Cayo et al. 2012) and correct these disorders (Yusa et al. 2011, Fattahi et al. 2013) both in vitro and in vivo. While differentiated HLCs hold lots of potential for reversal of some conditions others such as cirrhosis can only be treated by transplant, requiring whole organs. In contrast to the scarcity of organs for human transplant PSC derived organoids represent a potentially unlimited source of organs, if embryonic development on a single organ scale can be successfully imitated ex vivo. Such structures could also be used in biomedical research to investigate disease and drug toxicity, with the added advantage of using human cells in models where animal cells are often currently used.

In vivo the polarity of hepatocytes and their arrangement with ECs and BECs in a 3D architecture are necessary for both the endocrine and exocrine functions of the liver (Si-Tayeb et al. 2010). To fully recapitulate liver functions from PSCs may also require a mixed population of hepatic cells, with correct polarity and arrangement into 3D structures. 3D culture techniques have been shown to support hepatocytes in a differentiated state (Sivaraman et al. 2005) for a longer time period compared to monolayer culture (Rowe et al. 2013) and may offer the same advantages to PSC models.

A successfully differentiated organoid will more closely mimic the multi cellular complexity and 3D architecture crucial to the function of the liver, including cell to cell and cell to ECM interactions. Allowing the mechanics involved in disease and injury to be investigated in whole organs in vitro in a system similar to organ slices already used to model liver toxicity and fibrosis (Van de Bovenkamp et al. 2007, Hadi et al. 2013).

Whole mount imaging can be used to reveal detailed morphological data. It allows images to be taken as curved cross sections as well as flat planes and can be used to follow individual cells, 2D cell layers and 3D structures on any axis and over different depths. To assess whether the complexity of liver development and structure can be faithfully recapitulated in vitro we cultured a mixed mesoderm and endoderm population of ESC derived cells in a 3D air to liquid interface system. Building on the previous monolayer hepatocyte from hESC differentiation protocol and relying on the self-organising capacity of differentiated cells. Organoids were then visualised by whole mount microscopy and compared to human fetal liver tissue of various developmental stages. This resulted in differentiated hepatic cell types of both mesoderm and endoderm lineages organised into 3D structures reminiscent of liver architecture.
3.3 Materials and Methods

3.3.1 Human embryo collection and classification
The collection and use of human embryonic and fetal material was carried out following ethical approval issued by the Southampton & South West Hampshire Joint Local Research Ethics Committee and the North West Research Ethics Committee, under guidelines issued by the Polkinghorne committee. Written consent for the use of embryos was obtained from women undergoing termination of pregnancy. Human embryos were collected following medical or surgical termination, and staged by stereomicroscopy according to the Carnegie classification and the University of New South Wales embryology resource [http://embryology.med.unsw.edu.au/]. Fetal material was obtained following termination and foot length measured to give a direct estimate of fetal age as wpc. This work was carried out by the Endocrinology and Diabetes Group, Faculty of Medicine, the University of Manchester. Livers were dissected from embryos aged 12-20 weeks by Dr A. Berry

3.3.2 Cell culture

3.3.2.1 Fetal liver explant culture
Explants for DAPM were 16wpc and cultured in ALI3D system (Rowe et al. 2013). Livers were further dissected and units of tissue of roughly 20mg were placed on an OrganDOT membrane, hydrophilic polytetrafluoroethylene (PTFE) membrane discs (6 mm diameter, 0.4 μm thick; BioCell Interface SA), inside a 0.4 μm Millicell-standing cell culture plate insert (Millipore, UK), with 3 OrganDOTs per insert. A single insert was placed inside of a 10cm culture dish containing 12ml of Williams E medium supplemented with; ITS 1%, L-glutamine 1% (Sigma Aldrich, UK), Pen/strep 1% (PAA) and dexamethasone 100nM (Sigma Aldrich, UK) and incubated at 5% CO₂ and 37°C. The medium was changed daily and aspirated and replaced from underneath the insert only.

3.3.2.2 Stem cell culture
The HUES7 stem cells were cultured at 37°C, 5% CO₂ in medium containing; 458ml knockout D-MEM (Gibco), 120ml Knockout SR (Invitrogen), 6ml NEAA (PAA), 6ml L-glutamine, 6ml ITS-G supplement (Gibco), 3ml Pen/Strep, 1.1ml 2-mercaptoethanol 50mM (Gibco), 60μl FGF2 (Peprotech).
To passage hES cells, medium was first aspirated from the culture. Recombinant trypsin (Tryple Express, Life Technologies, UK) was added to cover the cells, 1ml per 1x6w plate, which was then incubated for 1-2 mins at room temperature. hES cells were observed under a microscope, and left until the edges of the colonies started to dissociate and the feeders started to pull away. Then PBS was used to wash the flask to ensure all cells were in suspension. Cells were transferred to a centrifuge tube and spun at 600g for 3 mins after which the supernatant was aspirated. The pellet was resuspended in hES medium and split at an appropriate ratio.

3.3.2.3 MEF culture

MEF cell culture was carried out in medium containing 500ml high glucose DMEM with L-glutamine, 5ml Pen/Strep, 50ml FBS (Life Technologies, UK). Medium was changed every other day.

To passage culture medium was aspirated from the cells. Recombinant trypsin (Tryple Express, Life Technologies, UK) was added to cover the cells, 4ml to a T75, which were then incubated for 1-2 min at 37°C 5% CO₂. When adequately trypsinized cells will begin to detach after gently tapping the side of the flask. PBS was used to wash the flask to ensure all cells were in suspension. Cells were transferred to a centrifuge tube and spun at 800xg for 5 min after which the supernatant was aspirated. The pellet was resuspended in MEF medium and split at a 1:3 ratio.

MEFs were inactivated between P3 and P5. Mitomycin C (Sigma Aldrich, UK) was first reconstituted in PBS to make a 1mg/ml solution. The medium was removed from each flask of cells to be inactivated and replaced with fresh medium containing 10μg/ml of mitomycin C. After incubation for 3 hours at 37°C the medium was aspirated and the cells washed with PBS. Cells were then dissociated as described in the MEF passage protocol. At this stage inactivated MEFs were plated immediately or frozen for future use. Inactivated MEFs were plated onto 0.1% gelatine coated tissue culture surfaces and seeded at a density of 2.5x10⁵ cells per well of a 6 well plate.

3.3.2.4 Thawing

Cryovials (Sigma Aldrich, UK) were removed from liquid nitrogen. The bottom half of the cryovial was placed in a 37°C water bath to loosen the pellet. It was then transferred to a 15ml centrifuge tube and 10ml of culture medium was added drop wise while agitating the centrifuge tube. The cell solution was then centrifuged at 600xg for 5 min. The supernatant
was removed and the pellet resuspended in culture medium and then transferred to appropriate format well or flask.

3.3.2.5 Freezing cells

Following trypsinisation and pelleting as above cells were resuspended in 1ml of freezing media (10% Dimethylsulphoxide (DMSO) (Sigma Aldrich, UK) and 90% FBS). The cells were transferred to cryovials and frozen at a rate of 1°C/minute to -80°C in a cryo-freezing container (Sigma Aldrich, UK) and then transferred to storage in liquid nitrogen.

3.3.2.6 Differentiation experiments

To differentiate hepatic organoids 70% confluent monolayers hESCs in 6-well plate format were grown in a series of different culture mediums at 37°C and 5% CO₂:

- **Day1-2**: RPMI, activin A at 100ng/ml (Peprotech), WNT3a at 25ng/ml (R&D systems), 0.5% FBS, penicillin/streptomycin.  
- **Day3-4**: RPMI, activin A, 0.5% FBS, penicillin/streptomycin.  
- **Day 5-7**: HCM (Lonza), BMP2 at 20ng/ml (Peprotech), FGF2 at 30ng/ml (Peprotech).  
- **Day 8-10**: HCM, BMP2 at 20ng/ml, FGF2 at 30ng/ml, activin A at 100ng/ml, WNT3a at 25ng/ml.  
- **Day11-12**: HCM, HGF at 20ng/ml (Peprotech), activin A at 100ng/ml, WNT3a at 25ng/ml.  
- **Day13-15**: HCM, HGF at 20ng/ml.  
- **Day16-23**: HCM. Cells were in volumes of 3ml/day during stage 1 and 1.5ml/day in the other stages

For the organoid differentiation experiments cells were trypsinised at the end of day 4, centrifuged at 600xg and resuspended in 40ul of culture medium, plus rho-associated protein kinase inhibitor (ROCKi) Y-27632 (Sigma Aldrich, UK) at 1:1000, and reseeded onto an OrganDOT membrane, inside a 0.4 μm Millicell-standing cell culture plate insert (Millipore, UK). Medium was aspirated and replaced from underneath the insert only

For the HLC+W/A organoids; OSM at 10ng/ml (Sigma Aldrich, UK) and DEX at 100nm were added during **Day16-23**. For the HLC organoid WNT3a and activin A were omitted from **Day8-12** and OSM at 10ng/ml (Sigma Aldrich, UK) and DEX at 100nm were once again added during **Day16-23**.

3.3.3 DAPM toxicity assay

Varying masses of DAPM (Sigma Aldrich, UK) were diluted in appropriate amounts of DMSO and then added to HCM to result in final DAPM concentrations of 0mM, 0.5mM, 1mM, and 5mM, with a fixed 0.8% of DMSO in the culture medium.
3.3.4 Liver Function Tests

1.5ml cell culture medium for each organoid after DAPM treatment was vacuum centrifuged at 37°C, minimum pressure, until only 200µl of supernatant remained. Using a 200µl volume of concentrated cell culture medium LFTs quantified the presence of ALB, ALT, AST and GGT. LFTs were carried out on a Roche Cobas 8000 autoanalyser. The samples were assayed by Allison Gaskell of the Department of clinical biochemistry, Manchester royal infirmary.

3.3.5 Albumin ELISA

Albumin secretion was quantified using the Human Albumin ELISA Quantitation Set (Bethyl Laboratories Inc). 1µl of affinity purified antibody was added to 100µl of coating buffer for each sample. 100µl of diluted coating antibody was added to 1 well per sample of a 96 well plate. This was incubated at room temperature for 1 hr and then washed 5 times. To each well was added 200µl of blocking solution and the plate was incubated at room temperature for 30 mins. The plate was washed 5 times and 100µl of sample was added to each well and the plate was then incubated for 1 hr. After 5 more washes 100µl of diluted horseradish peroxidase (HRP) detection antibody was added to each well and the plate was incubated at room temperature for 1hr. The plate was washed 5 times and 100µl of TMB substrate solution was added to each well. The plate was then developed in the dark at room temperature for 15 mins after which the reaction was stopped by adding 100µl of stop solution to each well and the absorbance measured on a plate reader at 450nm. Results were quantified by comparison to a standard curve generated form known quantities of albumin. To generate values of albumin relative to mass fetal liver explants were individually weighed on the day of cell culture medium collection. Whereas organoids were assumed to be the average weight of 3.58mg (n=16).

3.3.6 Immunocytochemistry of monolayer experiments

Cells were fixed in 4% paraformaldehyde (Sigma Aldrich, UK) for 10 mins then washed three times in PBS. Blocking solution was prepared containing PBS, 0.1% triton x and 10% serum and added to the cells for 30 mins. Blocking solution was aspirated and a primary antibody solution added to the cells and left overnight at 4°C. Primary antibody solution consisted of; the antibody at its specified dilution, 10% serum as used in the block,
0.1% triton x (Sigma Aldrich, UK) and PBS. The primary antibody was then aspirated and the cells washed three times in PBS. All Alexa Fluor secondary antibodies (Life Technologies) were diluted 1:1500 in PBS with 0.1% triton x. The secondary antibody solution was applied for 2hr at room temperature in the dark. The secondary antibody was then aspirated and the cells washed three times in PBS. Nuclei were then stained with DRAQ5 (Biostatus) at 1:1000, a far red DNA dye, for a minimum of 30 minutes before being imaged.

3.3.7 Whole mount immunofluorescence and confocal imaging

OrganDOT cultures were submerged in 4% PFA for 45 minutes and then washed in PBS three times. Fixed samples were stored in PBS until required. Fixed OrganDOT cultures were blocked for 2x 45 minutes on a shaker in PBS containing 0.1% triton X 100 (PBS-triton) and 10% serum (incubation buffer), which matched the species the secondary antibody was raised in. Primary antibody was then diluted with incubation buffer and left on a shaker overnight at 4°C. The primary antibody was then removed and the sample washed for 2x 45 minutes in PBS-triton. All Alexa Fluor secondary antibodies were diluted 1:1500 in incubation buffer and added to the samples for 3-4 hours at 4°C on a shaker before being washed for 2x 45 minutes in PBS-triton. To dual label the samples the above steps were repeated. Nuclei were then stained with DRAQ5 at 1:1000, a far red DNA dye, for a minimum of 30 minutes before being imaged on a confocal.

Images were collected on a Leica TCS SP5 AOBS upright confocal using a 20x/0.50 Plan Fluor spher objective and varying confocal zoom. The confocal settings were as follows, pinhole 1 airy unit, scan speed 700Hz unidirectional, resolution 1024 x 1024. Images were collected using the following detection mirror settings; Alexa Fluor 494-565nm; Texas red 599-665nm; DRAQ5 690-800nm using the 488nm (30%), 594nm (100%) and 633nm (100%) laser lines respectively. To eliminate cross-talk between channels, the images were collected sequentially.

3.3.8 Tissue embedding, sectioning and immunohistochemistry

Following dissection tissue was fixed in 4% paraformaldehyde (Sigma Aldrich, UK) and agitated on a rocker at room temperature overnight. The tissue was dehydrated by sequential immersing in 70%, 80%, 90%, 100% ethanol (Fisher Scientific, UK) and then xylene (Genta Environmental, UK) for 2 hours each, at room temperature. Xylene was
removed from the tissue by washing in chloroform (Fisher Scientific, UK) overnight at room temperature with agitation. To embed tissue was incubated in three changes of paraffin (Fisher Scientific, UK), for two hours at 60°C; the final change was carried out under vacuum to remove any residual air bubbles. The samples were then placed in a cassette, embedded in paraffin and allowed to solidify before sectioning.

Paraffin blocks were mounted on a microtome (Leica RM2235, Leica Microsystems, Germany) and cut into ribbons of 5μm sections. Ribbons were placed onto boards and cut into individual sections. Each section was mounted onto an X-tra adhesive coated glass slide (Leica Microsystems, Germany) by floating on H2O at 45°C to expand the paraffin wax, H2O underneath the section was removed by aspiration. Slides were dried in an oven at 37°C overnight and then stained.

To de-wax, slides were washed 2x for 3 minutes in xylene followed by 2 minutes in 100% ethanol, 2 minutes in 90% ethanol then by a rinse in water. Slides were then incubated in 3% (v/v) H2O2 (Sigma Aldrich, UK) in PBS for 20 minutes to quench endogenous peroxidases, followed by three 5 minute washes in PBS. Antigen retrieval was carried out by incubating slides in 90°C 10mM sodium citrate (pH 6) (Fisher Scientific, UK) for 30 minutes. Slides were washed once in PBS. Primary antibody was diluted in PBS (pH 7.4) with triton-x and 3% serum of the animal the secondary antibody was raised in. Slides were incubated with the primary antibody overnight at 4°C.

Sections were washed in PBS 3x for 5 minutes before incubation with a biotinylated secondary antibody diluted in PBS-triton for 2 hours at 4°C. Slides were washed for 5 minutes in PBS three times and incubated with Horseradish peroxidise streptavidin (1:200, Vector Laboratories, UK) for one hour at 4°C. Slides were washed in PBS 3x for 5 minutes.

Sections were then incubated in 3,3′-Diaminobenzidine (DAB, Merck Chemicals Ltd, UK) with 0.03% H2O2 for 3 minutes to develop the colour reaction. Slides were washed in PBS and counterstained in toluidine blue (Sigma Aldrich, UK) for 2 minutes. Sections were dehydrated by incubations in 70% ethanol for 30s, 90% ethanol for 30s, 100% ethanol for 3 minutes and two incubations in xylene for 3 minutes each, slides were then mounted with a coverslip using entalan (Merck Chemicals Ltd, UK). Sections were analysed with Axiovision software using an Axio A1 Imager and Adobe Photoshop CS4 Software.
### 3.3.9 Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Company</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
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<td>anti mouse IgG</td>
</tr>
<tr>
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</tr>
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<td>anti mouse IgG</td>
</tr>
<tr>
<td>EPCAM</td>
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<tr>
<td>VEGF</td>
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<td>anti rabbit IgG</td>
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</tbody>
</table>

Table 3-1 Details of antibodies in immunofluorescence.

### 3.4 Results

#### 3.4.1 Differentiation of ESCs to liver organoids

A protocol based on our previous work to generate HLCs in monolayer (Baxter et al. 2014) was adapted to generate hepatic organoids containing hepatocyte- and cholangiocyte-like cells. We hypothesised 3D culture conditions would improve the differentiation of cholangiocytes as BEC formation in development is intricately linked with bile duct
morphogenesis and the 3D culture system may allow the cells to form polarity as in vivo, a property linked with cells orientation in structures like the bile ducts where the apical side of the cholangiocyte faces the lumen, and develop structures during their differentiation.

<table>
<thead>
<tr>
<th>Monolayer</th>
<th>Organoid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endoderm formation:</strong></td>
<td><strong>Organoid</strong></td>
</tr>
<tr>
<td><strong>DAY 1-2</strong></td>
<td>Hepatic maturation: DAY 11-15</td>
</tr>
<tr>
<td>0.5% FBS, 100ng/ml Activin A 25ng/ml Wnt3a</td>
<td>HCM + 20ng/ml HGF</td>
</tr>
<tr>
<td><strong>Endoderm formation:</strong></td>
<td>Hepatocyte maturation: DAY 16-24</td>
</tr>
<tr>
<td><strong>DAY 3-4</strong></td>
<td>HCM</td>
</tr>
<tr>
<td>0.5% FBS 100ng/ml Activin A</td>
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</tr>
<tr>
<td><strong>Hepatic specification:</strong></td>
<td>Portal signalling: <strong>DAYS 8-12</strong> 100ng/ml Activin A 25ng/ml Wnt3a</td>
</tr>
<tr>
<td><strong>DAY 5-10</strong></td>
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<tr>
<td>Lonza hepatocyte culture medium (HCM)</td>
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<tr>
<td>20ng/ml BMP2 30ng/ml FGF2</td>
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**Figure 3-1 Hepatic organoid differentiation protocol.**

A schematic showing the timing and composition of the differentiation medium

The protocol aims to mimic stages seen during in vivo development with the addition of soluble factors (Fig. 1). Beginning in monolayer with addition of activin A, WNT3a and low serum (PI3K inhibition) to the ESCs, to generate definitive endoderm (DE) like cells as demonstrated in other protocols (D’Amour et al. 2005, Cai et al. 2007, Nakanishi et al. 2009, Zhao et al. 2009). In vivo studies resulted in a model where TGFβ signalling controls the hepatic induction of endoderm by BMP and FGF signalling, restricting cells to an endoderm fate until the appropriate time (Wandzioch and Zaret 2009). This fits well with the protocol where activin A, a member of the TGFβ superfamily, is added to the culture medium until all the cells have a DE morphology. WNT3a is removed after 2 days to encourage an anterior endoderm identity which in vivo requires WNT inhibition (McLlin et al. 2007). As shown previously at day 4 in the protocol the resulting cells express the DE associated transcription factors FOXA2, SOX17 and GATA4. In the HUES7 cell line used for organoid experiments 87% were FOXA2+, 84% SOX17+ and 96% were GATA4+ (Baxter et al. 2014). The discrepancy in the number of cells positive for each transcription factor suggests a population of roughly 10% GATA4+/FOX-ve/SOX-ve cells which may represent a lateral mesoderm population which are also GATA4+ in vivo (Rojas et al. 2005).
Organoid culture system.

Image showing the organoid culture system. Organoids are shown on top of PTFE membranes which themselves are on a cell culture insert. Medium is added underneath the cell culture inserts.

The DE-like cells are then dissociated and seeded on an organDOT membrane and cultured at the air liquid interface (Fig. 2), a technique shown to enhance and prolong the phenotype of cultured fetal hepatocytes (Rowe et al. 2013). At this point the cells are cultured with the addition of FGF and BMP for 6 days to mimic the formation of the liver bud in vivo (Jung et al. 1999, Rossi et al. 2001, Chung et al. 2008). After 3 days, WNT3a and Activin A were added to the culture medium for 5 days overlapping with both the FGF/BMP containing medium and the subsequent stage. WNT has been shown to have a role in both the formation of the liver bud (Ober et al. 2006, Goessling et al. 2008) and cholangiocyte differentiation (Decaens et al. 2008, Tan et al. 2008) while activin A, is found at high levels in the portal area of the developing liver and controls cholangiocyte differentiation (Clotman and Lemaigre 2006, Antoniou et al. 2009). The addition of HGF to the medium for 5 days followed the FGF/BMP stage. HGF is known to cause the proliferation of hepatic progenitors (Apte et al. 2006) and in combination with EGF, which is found in the base medium, has been shown to drive the formation of cholangiocyte containing 3D duct and cyst structures in ECM based gels (Tanimizu et al. 2007). The experiments were continued for a further 7 days in the base medium. Cortisone has been found to be necessary for growth and differentiation of fetal hepatoblasts (Bennett et al. 1987) its derivative cortisol is also present in the base medium.
3.4.2 Differentiation of cholangiocyte-like cells and their formation of duct-like structures in the 3D culture system

CK7 marks cytokeratin filaments in the cytoplasm. Immunostaining of hepatic organoid cultures at the end of the protocol shows the organisation of CK7+ cells into structures with a circular cross section surrounding a central lumen, a marker and morphology shared with cholangiocytes and biliary ducts in vivo (Fig. 3a-f, and 4). These CLCs are HNF4α-ve and are a separate population from the HNF4α+ HLCs which are also present within the organoid (Fig. 3a). In Figure 3a the two cell types are shown separated from each other by a population of cells negative for both of their respective markers.

The CK7+ cells coexpress epithelial cell adhesion molecule (EPCAM) (Fig. 3b) a cell surface marker expressed in biliary ducts during development (Fig. 4).

In vivo laminin surrounds the basal side of biliary ducts (Carpentier et al. 2011, Espanol-Suner et al. 2012). In the organoid laminin surrounds the basal pole of some of the cholangiocytes in the duct like structures (Fig. 3c) showing both the secretion of ECM de novo and formation of cell polarity. The CLCs of this particular duct-like structure (DLS) show the square morphology seen in BEC in vivo, (Yamasaki et al. 2006).

CK7+ DLS with smaller lumens are also seen which stain positive for the transcription factors HNF6 and HNF1β (Fig. 3d, e), both markers of hepatoblasts and cholangiocytes, whilst also being surrounded by cells positive for these markers but CK7-ve. HNF6+/CK7+ cells in the organoid (Fig. 3d) have the appearance of the developing duct stage when lumen formation first occurs between CK+ and CK-ve cells, around E16.5 in mice (Coffinier et al. 2002). HNF1β expression is also found in both the early parenchyma and BECs (Barbacci et al. 1999, Coffinier et al. 2002). The cells surrounding smaller DLS are still HNF1β+ (Fig. 3e), unlike duct surrounding parenchymal cells in vivo, suggesting the persistence of ductal plate inducing or fetal signalling. Alternatively these CK7-ve/HNF1β+ cells could be CK19+/CK7-ve cholangiocytes.

Some DLS (Fig. 3f, arrowhead) are heterogeneously positive for CK7 showing possible induction of this marker over time which in vivo occurs as the cells mature (Van Eyken et al. 1988). The CLC structures and surrounding cells are positive for the growth factor VEGF in the organoid (Fig. 3f), as are the BECs, ECs and hepatoblasts in vivo (Morell et al. 2013, Walter et al. 2014).

The CLC also co-expressed the markers SOX9 and CK19 (Fig. 3g). SOX9, a transcription factor, is one of the earliest markers of bile duct formation (Antoniou et al. 2009) while CK19 is found in both hepatoblasts and cholangiocytes. Both proteins are expressed throughout development and in the adult biliary ducts (Fig. 4).
Immunostaining of monolayer experiments after the same differentiation protocol revealed no organisation of CK7+ cells or lumen (Fig. 3h). CK7+ cells had a different morphology in monolayer when compared with organoid culture (Fig. 3i). The latter better resembled in vivo cholangiocytes with nuclei almost immediately adjacent to each other separated by a dense line of CK7 staining; the monolayer cells had much larger cytoplasmic areas and large keratin networks spread throughout the cells. Monolayer CLCs still co-expressed the CK7 and HNF1β characteristic of BECs as well as cultures having HNF1β-only positive cells, potentially indicative of earlier staged BECs or hepatoblasts (Fig. 3h).

Figure 3-3 Expression of cholangiocyte markers and formation of DLS in the organoids.

Whole mount immunofluorescence of fixed organoids (a-g) and monolayer (h, i) at day 23. a, CK7/HNF4α dual immunofluorescence reveals separate populations of cholangiocyte-like and hepatocyte-like cells. b, These CK7+ cells co-express EPCAM. c, CK7+ cells are arranged around a central lumen and are in contact with a laminin basement matrix. d, e, CK7+ cells co-express HNF6 and HNF1β. f, The DLS show areas of VEGF expression. g, DLS co-express CK19 and SOX9. h, Monolayer
control experiments also show co-expression of CK7 and HNF1β but no organisation of these cells into structures. Individual CK7+ cells in monolayer experiments have a different morphology than those of the organoids, with larger cytoplasmic and more diffuse cytokeratin filaments. Scale bars: 50µm, (c-e,g,i). 100µm, (a,b,h). 200µm, (f). Organoid staining representative of n>5 experiments, except VEGF where n=3. Monolayer staining representative of 3 experiments.

Staining of primary human tissue (Fig. 4) revealed EPCAM expression; in hepatoblasts with stronger expression in the portal regions at 8wpc, in the ductal plate and developing ducts at 16wpc while reduced in the parenchyma [but still positive at 18wpc as shown by immunofluorescence (Fig. 5g)], and absent in the adult liver. CK7 staining was absent at 8wpc and 16wpc but positive in the adult cholangiocytes, showing its use as a marker of more mature duct structures and cholangiocytes. CK19 and SOX9 were both found expressed in the cells of the ductal plate and in the cholangiocytes at 8wpc, 16wpc and adult livers; showing their use as cholangiocyte markers throughout development.
Figure 3-4 Cholangiocyte markers during human development.

Immunostaining of primary human liver tissue in 5µm sections showing changes in cholangiocyte markers and structures during development. EPCAM staining is seen throughout the liver at 8wpc before becoming concentrated in the ductal plate and bilayered ductal structures by 16wpc, it is then absent in the adult liver. CK7 staining is absent until after 16wpc throughout the liver but cholangiocytes are strongly positive in the adult. CK19 and SOX9 mark the ductal plate and cholangiocytes at all time points stained. Scale bars: 100µm.
3.4.3 Differentiation of a heterogeneous population of HLCs with both postnatal and fetal markers, distinct from the CLCs

Hepatocyte markers were found to be expressed in the organoid cultures. Large areas within the organoids contained cells stained for HNF4α (Fig. 5a-e), a transcription factor which marks hepatocytes in vivo. HNF4α drives hepatocyte differentiation (Li et al. 2000); it is expressed in the developing hepatoblasts from E8.75 in mice (Duncan et al. 1994, Taraviras et al. 1994) and continues throughout development. It is lost in developing cholangiocytes (Yamasaki et al. 2006) and is also absent in PC adult hepatocytes (Stanulovic et al. 2007). EPCAM expression is found localised to the surface of cells in the organoids (Fig. 5a). All HNF4α+ cells co-express EPCAM with most but not all EPCAM+ cells co-expressing HNF4α. The EPCAM+/HNF4α-ve cells could represent CLCs as both cholangiocytes and hepatocytes express EPCAM at early stages of development. Staining in the embryo also shows EPCAM expression in the kidney and pancreas (Fig. 5j) but not elsewhere. In vivo EPCAM marks the parenchymal cells of the liver during development from at least as early as Carnegie stage 17 (CS17) (Fig. 5k) and is still present at lower levels at 18wpc (Fig. 5g) but is expected to be absent in the adult liver (de Boer et al. 1999). At 18wpc it is seen localised at the cell surface and shows higher expression levels in venous areas (Fig. 5g). The strongly EPCAM+ HLCs suggest relative immaturity.

The majority of HNF4α+ cells co-express CPS1 (Fig. 5b), an enzyme involved in the urea cycle found in the liver in vivo (Haussinger et al. 1992). CPS1 is expressed in the fetal liver where it is not yet restricted to periportal areas (Moorman et al. 1989). It is shown in the 18wpc liver staining the cytoplasm of all parenchymal cells including the venous area, indicating the lack of zonation (Fig. 5h).

Cytoplasmic GS expression is found in the organoid (Fig. 5c), mostly within HNF4α+ cells though there are small regions of GS+/HNF4α-ve cells. HNF4α+ cells show GS co-expression at both high and low levels. It is an enzyme involved in ammonia detoxification (Haussinger et al. 1992) absent in the human fetal liver (Moorman et al. 1989) as shown at 18wpc (Fig. 5i, the green channel shows the autofluorescence of the hepatocytes).

Hepatocytes at birth are arranged in cords that are still several cells thick as opposed to the adult which is one cell thick (Moorman et al. 1989). The organoid hepatocytes lack this cord structure forming layers instead (Fig. 5a-c, e).

In vivo MRP2 marks the bile canaliculi of polarised hepatocytes, expressed on their apical membrane. In the organoid MRP2 expression was found in channel shapes extending for several cell lengths past HNF4α+ cells analogous to canaliculi (Fig. 5d). Showing that they, like the CLCs, exhibit polarity.
HNF1β is also expressed (Fig. 5e) which marks early hepatocytes/hepatoblasts and later the cholangiocytes (Barbacci et al. 1999, Coffinier et al. 2002). Co-staining with HNF4α reveals that the majority of cells are HNF1β low or –ve while some express both transcription factors and some cells are HNF1β+/HNF4α-ve, these cells could be interpreted as hepatocytes, hepatoblasts and cholangiocytes respectively.

HNF6 is found to be expressed in the organoids (Fig. 5f). In vivo HNF6 marks the early hepatocytes and expression is heterogeneous throughout the parenchyma at E15.5 and increased in the ductal plate (Clotman et al. 2002). In the organoid most of the parenchymal-like cells are HNF6-ve. Some HNF6+/CEBPα–ve cells could be CLCs, or represent the heterogeneous expression seen in the developing liver parenchyma.

The transcription factor CEBPα marks hepatocyte progenitors throughout development from at least as early as E12.5 in mice and is still expressed at birth (Yamasaki et al. 2006) and is found in cell nuclei in the organoid (Fig. 5f). CEBPα expression inhibits HNF6 (Rastegar et al. 2000), and hence CEBPα inhibition is necessary for cholangiocyte formation (Yamasaki et al. 2006). In the organoid some CEBPα cells co-express low levels of HNF6 which could be interpreted as either the beginning of cholangiocyte specification or the regression of ductal plate like cells to a hepatocyte cell type.

The presence of HNF1β, HNF6 and EPCAM in the HLCs indicate a fetal rather than adult developmental stage.
Figure 3-5 Expression of hepatocyte markers in organoids and fetal liver.

Whole mount immunofluorescence of fixed organoids at day 23 (a-f) revealed hepatocyte-like cells co-expressing the markers; EPCAM and HNF4α (a), CPS1 and HNF4α (b), GS and HNF4α (c). d, shows the presence of the apical marker MRP2 expressed in channels between cells. HNF4α and HNF1β are expressed by overlapping populations of cells (e) as are CEBPα and HNF6 (f). Immunostaining of the liver parenchyma, whole mount (g-i) and 5µm sections (j, k), revealed EPCAM expression by parenchymal cells from CS17 until at least 18wpc and CPS1 expression throughout the parenchyma at 18wpc. Scale bars: 50µm, (a,c,d). 100µm, (b,f,i,k). 200µm, (e,g,h). 2mm, (j). Organoid staining representative of n>5 experiments, except for MRP2 staining where n=3.
3.4.4 Cells with mesothelial morphology and marker expression are found at the organoid periphery

The liver has an outer mesothelial lining and the ESC derived organoids also express MC markers in their outer layer. Cells positive for CK7 (Fig. 6a), NCAM and DES (Fig. 6c, d) are found as a single layer surrounding the outside of parts of the organoids. Both NCAM and DES are seen in the cytoplasm while NCAM in addition can be seen concentrated at the cell surface. These CK7+ cells (Fig. 6a) are spaced further apart than the CLCs of the duct-like structure seen adjacent and lack their squamous morphology, instead they have the appearance of beads on a string. The morphology and staining of these cells resemble the mesothelial lining of the liver. In vivo this is a single cell layer of MCs that are also laminin positive (Li et al. 2013). Laminin an ECM protein was also found to surround the organoids (Fig. 6b).

Staining of primary fetal tissue (Fig. 6e) shows a single layer of narrow cells surrounding the liver, positive for the epithelial cell marker CK19 from CS17 (or earlier) throughout development and in the adult liver. While staining for CK7 is initially negative in the liver these cells begin to co-express CK7 and CK19 between 8 and 16wpc. This gain of CK7 expression by CK19+ cells during development is also seen in the biliary ducts though seemingly at a later time point. Also visible in the top left image of Figure 6e is the adjacent STM surrounded by its own parietal mesothelium which is also CK19+.

The MCs in the mouse at E12.5 are panCK+/DES-ve and by E15.5 they have gained DES+ expression (Asahina et al. 2009, Asahina et al. 2011). In humans MCs were NCAM+ until 8wpc but not after and DES+ from 7wpc until at least 20wpc (Loo and Wu 2008). Staining in the organoid shows an outer layer of cells in one area that are NCAM+/DES-ve (Fig. 6d) suggesting an earlier stage of development than other areas (<7wpc) (Loo and Wu 2008), or perhaps a parietal mesothelial equivalent.
Figure 3-6 Expression of mesothelial markers at the periphery of organoids and fetal liver.

Whole mount immunofluorescence of fixed organoids at day 23 (a-d). Staining reveals an outer layer, one cell thick, surrounding parts of the organoid. These cells
have a mesothelial morphology and are positive for the markers CK7 (a), laminin (b) and DES (c). d, shows a cell with a narrow cytoplasm that is NCAM+/DES-ve, a profile seen in early liver MCs. Immunostaining of primary liver tissue (e) shows the expression of cytokeratins in liver mesothelial cells. CK19 is present from CS17 throughout development and in the adult liver. CK7 only begins to be expressed by these cells between 8wpc and 16wpc. Scale bar: 50µm, (a,d,e). 100µm, (b,c). Organoid staining representative of n>4 experiments except laminin where n=3.

3.4.5 Differentiation of cells with liver specific endothelial markers; their organisation into structures and its dependence on an organoid culture format

CD34 is an endothelial cell marker expressed in continuous microvasculature (Scoazec and Feldmann 1991). CD34 in the organoids marks branching structures, reminiscent of microvasculature, of varying sizes (Fig. 7a-c, e, f). Figure 7a, which covers a large z volume, shows the formation of circular shapes by the branching structures, these are also seen within the sinusoids of the fetal liver (Fig. 7i). Figure 7b is a cross-section which shows the increased localisation of CD34, a cell surface marker, to the cell membranes and junctions in comparison to the cytoplasm. It also reveals the presence of circular gaps in the CD34+ structure (arrowheads). These intracellular circular structures could be interpreted as the onset of lumen formation which when examined in vivo and vitro was found to occur by the fusion of intra- and intercellular vacuoles (Kamei et al. 2006). In Figure 7b two intracellular circular structures can be seen fusing in just this way. They also bear a resemblance to the contractile rings of dividing ECs (Phng et al. 2013).

Co-expression of CD31, a marker of continuous endothelium (Gouysse et al. 2002) localised to the cell surface, is found in the CD34+ vasculature structures (Fig. 7c, f). Not all areas of the CD34+ vasculature expressed CD31 and there were no CD31-only positive vasculature structures. It is also absent from the areas with intracellular circular structures (Fig. 7c), which may support these areas being discontinuous endothelium, as expression of CD31 is increased in sinusoids undergoing capillarization which become continuous.

LYVE1, a receptor found on discontinuous ECs (Lalor et al. 2006), is expressed in the LSECs from E14.5 in the developing liver of mice (Nonaka et al. 2007). LYVE1 is partially co-expressed by vasculature structures in the organoid (Fig. 7d-f). Co-staining with CD31 shows the two markers expressed in adjacent cells in the same structure (Fig. 7d, f). CD34/LYVE1 co-staining revealed co-expression, with all LYVE1+ areas also CD34+ (Fig. 7e, f) including the intracellular gaps (Fig. 7e). Triple labelling of the three markers CD31/CD34/LYVE1 shows that CD34 is expressed throughout the structures whereas LYVE1 and CD31 are expressed in adjacent areas with small areas of overlap (Fig. 7f).
Also visible are multiple fine projections, with the appearance of filopodia, extending from the cytoplasm of CD34+ and LYVE1+ cells (Fig. 7b, f). In vivo filopodia are found in abundance on highly polarised ECs, known as endothelial tip cells, during sprouting angiogenesis. They extend in the direction of migration (Gerhardt et al. 2003) where they interact with the cell matrix and other cells (Mattila and Lappalainen 2008, Arjonen et al. 2011). Figure 7f in particular shows a small EC structure which supports the idea that it would be undergoing angiogenesis.

In monolayer experiments (Fig. 7g) individual CD34+ cells are present with small angular cytoplasms. They do not organise themselves into structure or groups, nor show filopodia. The cells of the monolayer experiment were CD31-ve.

Staining of the endoderm stage cells (Fig. 7h) reveals low levels of CD34 expression, and separate CD31 labelling in different cells. Co-expression of CD34/CD31 could be interpreted as staining the hemangioblast – the endothelial and hematopoietic cell precursor. The lack of co-expression and the low amounts of CD34 suggests that this cell type is not present at this stage, if at all.

Staining of the fetal liver at 18wpc (Fig. 7i) shows LYVE1 expression localised to the sinusoids which are spread throughout the parenchyma. CD34 is expressed by the larger vasculature which maybe the central vein still expected to be CD34+ at this point in development (Gouysse et al. 2002). It is co-expressed with LYVE1 by the sinusoids in the areas where these two vasculature networks anastomose. This shows the majority of LSECs have lost CD34 expression in vivo by 18wpc.
Figure 3-7 Expression of endothelial markers in organoids, monolayer and fetal liver.

Whole mount immunofluorescence of fixed organoids (a-f) and monolayer cells (g) at day 23, as well as endoderm stage cells (h) at day 5. a, Imaging over a large Z volume shows branching and circular vascular structures similar to the sinusoids of the fetal liver, positive for the endothelial marker CD34. b, A single layer, shows intracellular gaps/lumen (arrowheads) and filopodia are present. c, Dual staining with CD34 and CD31 shows areas of co-expression. d-f, Structures were found to express LYVE1 an LSEC specific marker. Multiple labelling of LYVE1/CD31 (d), LYVE1/CD34 (e) and LYVE1/CD34/CD31 (f) show adjacent CD31 and LYVE1 positive cells both expressing CD34. LYVE1 staining also highlights intracellular gaps/lumen (e) and marks filopodia (f). g, Immunofluorescence of monolayer cells reveals no CD31 staining and no structure or organisation in the CD34+ cells. h, Immunofluorescence of monolayer endoderm stage cells showed no CD34/CD31 dual positive cells. i, Whole mount immunofluorescence of primary human fetal liver at 18wpc. LYVE1 staining is shown throughout the sinusoids but not in the veinous endothelial cells which are CD34+. Over a large Z volume LSECs form circular structures. LYVE1/CD34 co-expression is seen where the sinusoids connect to the larger vasculature structures. Scale bars: 25µm, (f). 50µm, (b-e,g,h). 100µm, (a). 200µm, (i). Organoid staining representative n>5 experiments, monolayer and endoderm staining representative of 3 experiments.
3.4.6 Cells positive for macrophage/monocyte markers are present in the organoid

CD68 is a glycoprotein and lysosome membrane protein highly expressed on tissue macrophages and monocytes (Parwaresch et al. 1986, Micklem et al. 1989, Holness and Simmons 1993). CD68 expression is found within the organoid (Fig. 8a-c). Figure 8a shows CD68 expression localised to circular organelles within the cytoplasm, possibly the endosomes or lysosomes. These CD68+ structures are DRAQ5-ve indicating the lack of cytoplasm in these specific areas in cross section. The CD68+ cells are all clustered together which could be a sign of their recruitment to a specific area or the site of their differentiation.

Kupffer cells, the liver specific macrophage, co-express CD31 (Lalor et al. 2006), a protein also expressed on the surface of monocytes and ECs (McMichael 1987, Favaloro et al. 1989). Co-staining with CD68 (Fig. 8b, c) revealed cell surface expression of CD31 on some CD68+ cells which together is suggestive of monocytes/macrophages. These CD68+ cells were also found in clusters but had a general cytoplasmic expression rather than localised to organelles.

Staining of CD68 in 18wpc fetal liver (Fig. 8d) shows its expression on the Kupffer cells, here too it is expressed throughout the cytoplasm. These cells have a different morphology from the CD68+ cells within the organoid suggesting that those are not fully developed Kupffer cells. Co-staining with LYVE1 shows they are localised to the sinusoids and are not clustered together.
Figure 3-8 Expression of macrophage/monocyte markers in organoids and fetal liver.

Whole mount immunofluorescence of fixed organoids at day 23 (a-c) and 18wpc fetal liver (d). a, CD68 is found to be localised to circular structures within the cytoplasm of cells of the organoid (inset), consistent with localisation to lysosomes or endosomes as found in monocytes and macrophages. b and c, CD68/CD31 co-staining revealed cells which express CD68 throughout the cytoplasm and CD31 on the cell surface, as found on macrophages and other white blood cells. d, CD68+ Kupffer cell located in the LYVE1+ sinusoids of fetal liver. Scale bars: 50µm. Organoid staining representative of 3 experiments.

3.4.7 Distinct populations of mesenchymal cells analogous to; hepatic stellate cells, myofibroblasts, pericytes, and mesenchyme of the liver are found in the organoid

In the liver specific microvasculature the LSECs are adjacent to the HSCs (Friedman 2008), a perisinusoidal cell, which shares many of the functions and markers of pericytes including PDGFRβ expression (Henderson et al. 2013).

Co-staining of the organoids with the markers CD34 and PDGFRβ (Fig. 9f) revealed PDGFRβ localised to the cytoplasm and surface of cells wrapped around and surrounding the CD34+ endothelial structures, with no co-expression. In vivo PDGFRβ is a cell surface receptor found on pericytes (Hellstrom et al. 1999), these cells surround and interact with the ECs of vasculature structures (Sims 1986). Showing that some EC structures of the organoid like those found in vivo are surrounded by specifically marked perisinusoidal
cells. This high magnification cross section of the cells reveals a large vacuole in the CD34+ structure, potentially a sign of lumen formation.

Cells with the morphology of HSCs, spindle shaped with long processes are seen in the organoid (Fig. 9a-e), these have DES+ cytoplasms a marker of HSCs during development (Loo and Wu 2008). Co-staining of DES and PDGFRβ (Fig. 9e) showed no co-expression of these markers which may indicate the presence of separate populations of vascular supporting cells.

αSMA is localised to the cytoplasm of myofibroblasts in vivo (Schmitt-Graff et al. 1991), as well the SMC of arteries (Asahina et al. 2011). In the organoid all the DES+ cells co-expressed αSMA in their cytoplasm (Fig. 9c, d), also seen were a separate population of larger DES-ve/αSMA+ cells which were fewer in number and had the stretched appearance of myofibroblasts (Fig. 9c).

NCAM marked the cytoplasm of a smaller population of cells with a similar morphology to the DES+ ones (Fig. 9a) these cells physically overlapped but no co-expression was seen. In vivo NCAM is found in early human fetal HSCs (Loo and Wu 2008).

SOX9, a transcription factor expressed by activated HSCs (Hanley et al. 2008), is co-expressed in a small number of DES+ cells in the organoid (Fig. 9b).

Figure 3-9 Characterisation of mesenchymal cells in the organoid.

Whole mount immunofluorescence of fixed organoids at day 23. DES staining was found to localise to the cytoplasm of cells with a mesenchymal morphology (a-e). a, Co-staining of DES and NCAM revealed separate populations of physically overlapping cells but no co-expression. b, A minority of DES+ cells were also
SOX9+. c and d, αSMA was co-expressed by all DES+ cells and also marked DES-ve cells with a fibroblast appearance (e). e, No co-expression of DES and PDGFRβ was seen. f, PDGFRβ+ cells were found to surround the CD34+ endothelial structures. A vacuole shows the CD34+ cells appear to be in the process of lumen formation. Scale bars: 25µm, (b,d,f). 50µm, (a,e). 100µm, (c). Staining representative n>4 experiments.

3.4.8 High doses of the toxin DAPM induce markers of hepatic damage while both high and low doses damage CLCs and DLS in the organoids

4,4′-Diaminodiphenylmethane  (DAPM or 4,4′-Methylenedianiline) (Fig. 10a) is a compound which results in general hepatic damage when given at high doses in rat. At lower doses/kg it specifically damages, and induces necrosis of, the biliary ducts of the liver leading to shedding of the epithelial lining into the lumen. While at high doses it damages both hepatocytes and cholangiocytes (Kanz et al. 1992, Kanz et al. 1998). The compound is metabolised by the liver leading to short lived reactive metabolites which are thought to be the cause of the injury (Kanz et al. 1995). This is hypothesised to be CYP mediated metabolism by the hepatocytes (Bailie et al. 1993). Examination of other body tissues after DAPM administration revealed no damage. Toxic effects are seen rapidly <24hrs and regeneration has been to shown to occur beginning at 72hrs. The resistance to damage of hepatocytes has been hypothesised to be due to glutathione mediated protection (Kanz et al. 2003).

Fetal liver explants cultured in an air-liquid interface 3D (ALI3D) system (Rowe et al. 2013) were treated with DAPM, at 5mM and 10mM doses, in the culture medium for 24 hours. Subsequent CK7 staining (Fig. 10c-e) showed disruption of the bile ducts, loss of tight junctions, and shedding of the epithelial lining into the lumen as described in the livers of in vivo DAPM treated rats. The DMSO treated control shows unaffected intact CK7+ bile ducts (Fig. 10b).

ESC derived organoid cultures also showed damage and disruption to the CK7+ structures and cells (Fig. 10g) after culture in DAPM containing medium. The previous condensed cytoskeletal localisation of CK7 has become diffuse through the cytoplasm as seen in comparison to the unaffected DLS of the vehicle control (Fig. 10f). Showing that these CK7+ cells behave in the same way as those of the fetal liver explants and the in vivo assays of the liver carried out by other groups. HNF4α expression was also lost in the 5mM DAPM treated organoid (Fig. 10g) suggesting that this is equivalent to the high doses given in animal models which was shown to affect the hepatocytes. CD34+ endothelial structures were still found in the 5mM treated organoids (Fig. 10h) showing that these cells are not disrupted by the compound, revealing its specificity.
Liver function tests (LFT) measure the release and function of liver enzymes and proteins into the blood supply in response to damage. Increased aspartate transaminase (AST), alanine transaminase (ALT) and decreased ALB are markers of hepatocyte damage while γ-glutamyl transferase (GGT) is indicative of biliary damage. LFT tests carried out on the organoid culture medium after DAPM treatment showed no significant change at a 1mM dose when compared to the vehicle control (DMSO) (Fig. 10i, j). At 5mM doses AST levels were significantly increased with a >3 fold change over the control and GGT levels rose from below the limit of detection (<0.46 U/L) to 0.78 +/- 0.06 U/L (mean +/- S.E.), signifying that at this dose the compound is hepatotoxic. ALT and ALB showed no significant change, though ALB levels did decrease in proportion to increasing DAPM concentration suggestive of hepatocyte damage. (The high albumin values in the medium are due to the detection of bovine serum albumin (BSA) in the medium using the LFT assay. This is not detected by the ELISA used to determine human albumin levels in Figure 13f and Figure 18c.)
3.4.9 Treatment with low doses of a cholangiocyte toxin induces hybrid hepatobiliary cells

ESC derived organoids treated for 24hr with DAPM at lower, 1mM and 0.5mM doses were examined. Assays using 1mM DAPM on the organoids, which had no measurable hepatotoxic effects (Fig. 10i, j), still showed damage to the DLS with CK7+ cells becoming disorganised and being shed into the luminal space (Fig. 11a-c), though the effects on CK7 localisation were not as severe as at 5mM as it was still localised to cytokeratin filaments. Revealing its cholangiocyte specific toxicity at this dose.

Effects on CK7+ cells were not apparent at 0.5mM (Fig. 11e, f). At both 0.5mM and 1mM HNF4α is still expressed (Fig. 11b-f) showing that the HLCs are not affected in the same way as at 5mM. However HNF4α+/CK7+ co-expression is seen which was absent from the vehicle control (Fig. 11c-e). This hepatocyte and biliary marker co-expression after DAPM treatment is analogous to the CK19+/HNF4α+ cells seen in rats treated with DAPM in vivo which has been interpreted as transdifferentiation of hepatocytes to cholangiocytes to regenerate damaged ducts (Limaye et al. 2010). These co-expressing cells in the organoid were located in proximity to a damaged DLS or found surrounding lumen.

Further signs of response to the DAPM treatment not seen in the DMSO control or untreated organoids include cells where CK7 expression is found on only the apical side as opposed to surrounding the cell (Fig. 11a, c, d) and individual rounded CK+ cells (Fig. 11a, c, e).
Whole mount immunofluorescence of fixed organoids at day 24 after 24 hr incubation with DAPM (a-f). a-c, CK7 staining reveals disrupted duct like structures at 1mM doses. b-f, HNF4α staining reveals the continued presence of HNF4α+ cells. c-e, Co-expression of CK7 and HNF4α represents a novel phenotype not seen in the control. e-f, 0.5mM doses of DAPM do not appear to disrupt the duct like structures. Scale bars: 50µm, (d,e). 100µm, (a,c). 200µm, (b,f). Organoid staining representative of 4 experiments.

3.4.10 The formation of cholangiocyte-like cells and their organisation into duct-like structures is not dependent on addition of WNT3a and activin A at Day8 of the protocol

WNT and activin A was hypothesised to be the cause for the differentiation of CLCs and formation of DLS structures in the hepatic organoids due to their roles in biliary differentiation in vivo (Hussain et al. 2004, Clotman et al. 2005, Clotman and Lemaigre 2006, Decaens et al. 2008, Tan et al. 2008). This was examined by immunostaining of organoids differentiated with the previously published HLC protocol (Baxter et al. 2014) both with and without the addition of WNT3a and activin A from Day8-12 (referred to as HLC+W/A and HLC respectively).

CK7+ duct like structures exist in both variations of the protocol (Fig. 12a, b). CK7+ cells still organise themselves into duct-like structures surrounding a central lumen, showing no change from the hepatic organoid protocol. Which shows that the attempt to induce portal signalling by the addition of WNT3a and activin A was not necessary to induce the
formation of CLCs and DLS. The inclusion of OSM and DEX in the HLC and HLC+W/A organoids, but absent in the hepatic organoid, had no noticeable effect on the formation of DLS structures from CK7+ cells.

CK7 staining in the HLC monolayer is also indistinguishable from that in the hepatic protocol monolayer cells (Fig. 12c).

3.4.11 Addition of WNT3a and activin A or OSM and DEX did not significantly affect hepatocyte characteristics and albumin organoid secretion relative to mass was comparable to that of the fetal liver

Due to the expression of fetal markers by the HLCs of the hepatic organoid the effects of addition of OSM and DEX to the protocol were examined as these have been shown to induce maturation of fetal hepatocytes in vivo (Kamiya et al. 1999, Kinoshita et al. 1999). Maturation of hepatocytes may then be evident in the expression of zonation markers. GS and CPS1 staining in the HLC+W/A organoid (Fig. 13d, e) revealed no qualitative change from the hepatic organoid. With the majority of HNF4α+ cells co-expressing both GS and CPS1, and with CPS1 expression being high in all cells whereas GS expression was heterogeneous. Some cells were shown to express HNF4α only.

Albumin secretion is a function of hepatocytes which increases with their maturation in vivo (Tilghman and Belayew 1982). Figure 13f shows the albumin secreted into the medium by the different protocol variations and by fetal liver explants as assayed by ELISA. The protocol variations show no significant effect on the HLCs when using albumin secretion as a proxy for their specification. The albumin secretion of the organoids
is between 50-66% of that of the ALI3D fetal liver culture when compared relative to mass. ALI3D shows the highest levels of albumin secretion in vitro for fetal liver cells (Rowe et al. 2013).

The action of WNT3a treatment on zonation markers was also examined by staining of the HLC protocol organoids in comparison to the HLC+W/A organoids, as WNT/β-catenin plays a role in hepatocyte zonation (Benhamouche et al. 2006, Burke et al. 2009, Colletti et al. 2009, Kaestner 2009). Staining in the HLC organoid again revealed no qualitative change in HLC zonal expression from the other protocol. There was varying levels of GS co-expression in HNF4α+ cells (Fig. 13a) with lots of dual positive cells forming clusters. Some cells were found to be HNF4α+/GS- and some were GS+/HNF4α-ve which may represent zonally separate HLCs, portal and central respectively. CPS1 co-expression was found in most but not all HNF4α+ cells in the HLC organoid (Fig. 13b), also found were CPS1+/HNF4α-ve cells with a different morphology (Fig. 13c).

Figure 3-13 HLC zonation markers in protocol variants and albumin secretion in comparison to fetal liver explants.

Whole mount dual-immunofluorescence of fixed organoids at day 23 (a-e), shows co-expression of GS/HNF4α and CPS1/HNF4α in both protocol variations. Individual cells positive for single markers are also seen. f. ELISA of cell culture medium shows albumin secretion from all three protocol variations with no significant difference between them, and from fetal liver explants. Error bars represent standard error, n=3-7 experiments. FL is fetal liver. Scale bars: 25µm, (e). 100µm, (c,d). 200µm, (a,b). Staining representative of 3 experiments.
3.4.12 Mesothelial markers surround the organoids in protocol variants

Examination of the HLC and HLC+W/A organoids using markers for MCs revealed no CK7+ outer layer of cells (Fig. 14a, c), unlike the hepatic organoid, though CK7-ve cells with the elongated and thin morphology of MCs were present (Fig. 14a). Cells of a mesothelial location and morphology in the HLC+W/A organoid co-express both DES and NCAM, MC markers seen earlier in development than CK7. HLC organoids are still positive for laminin (Fig. 14c), which here marks the outer edge of the organoid highlighting the absence of CK7 expression.

![Figure 3-14 Mesothelial markers in the protocol variations.](image)

Figure 3-14 Mesothelial markers in the protocol variations.

Whole mount immunofluorescence of fixed organoids at day 23. a, CK7-ve cells with a mesothelial morphology were seen in the HLC+W/A organoid. b, Dual staining reveals cells with a mesothelial morphology at the edge of the HLC+W/A organoid co-expressing the markers DES and NCAM. c, Staining of the HLC organoid shows no CK7+ cells but laminin is shown to mark the outer edge of the organoid. Scale bars: 50µm, (a,b). 100µm, (c). Staining representative of 3 experiments.

3.4.13 Addition of WNT3a and activin A induces formation of branching endothelial structures

Immunostaining in the HLC organoids reveals they have a qualitatively different localisation of EC markers (Fig. 15a, b) from the hepatic or HLC+W/A organoids. CD34+ cells do not form branching structures (Fig. 15a) like they do when treated with WNT3a and activin A (Fig. 15d). CD34 is instead localised to multi-vacuolated cells (Fig. 16a) and thin/angular cells (Fig. 15a) similar to CD34+ cells seen in the monolayer experiments with the appearance of individual ECs. These cells form no structures. Co-staining with CD31 (Fig. 15a) revealed no co-expression with CD34+ cells with only low levels of CD31 detectable anywhere in the organoid. LYVE1 staining in the HLC organoid (Fig. 15b) revealed a greatly increased number of cells positive for this marker. The morphology of these LYVE1+ cells is different to that of the CD34+ cells in this protocol suggesting
that they are marking different populations in these organoids. The LYVE1+ cells are in contact with each other (Fig. 15b, inset) but do not form organised branching structures.

Co-staining of the HLC+W/A organoids with the endothelial markers CD31 and CD34 (Fig. 15d) reveals large organised branching endothelial structures positive for CD34 throughout, with areas of co-expression of CD31. This is the same as the structures seen in the hepatic organoid. Multiple filopodia are again visible (Fig. 15d, inset). LYVE1 staining (Fig. 15e) revealed individual LYVE1+ cells as well as a small connected LYVE1+ structure though no large LYVE1+ branching structures were seen. The proximity of these LYVE1+ cells to each other suggests they could all be part of the same structure linked by cells positive for an unused endothelial marker. Comparison with the hepatic organoid shows the addition of OSM and DEX to the protocol has no obvious large scale effects on the vasculature cells that can be seen using these markers.

In the monolayer LYVE1 staining is present but stains fewer cells than in the organoid and appears globular rather than staining the whole cytoplasm (Fig. 15c).

Figure 3-15 Protocol variants endothelial markers.

Whole mount immunofluorescence of fixed organoids at day 23. a, Staining of the HLC protocol organoid revealed no CD34 positive vascular structures but cells with a thin angular morphology which do not co-express CD31. b, LYVE1 staining revealed large numbers of positive cells spread throughout the organoid. Inset shows the detail of LYVE1+ cell connections, also visible are small filopodia protruding from the LYVE1+ cells. c, LYVE1 staining in the HLC monolayer revealed no LYVE1+ cell networks and very few LYVE1+ cells. d, Staining of the combined protocol organoid shows CD34+ branching structures which partially co-express CD31. Inset shows the
detail of a CD34+ region, revealing multiple filopodia. e, LYVE1 staining reveals smaller areas of connected cells. Scale bars: 100µm, (c,d). 200µm, (a,b,e). Staining representative of 3 experiments.

3.4.14 Round vacuolated cells are seen in OSM and DEX treated organoids

In the HLC organoid CD34 is localised to cells with multiple large vacuoles which form no structures (Fig. 16a). CK7 staining shows CK7+ vacuolated cells seen in both protocols (Fig. 16b, c) treated with OSM and DEX (HLC and HLC+W/A organoids) absent from the hepatic organoid, they have the same morphology as the CD34+ cells also found in the HLC organoid. During human development CK7+/CD34+ cells have been identified in the liver, these were large with an elaborate cytoplasm (Suskind and Muench 2004).

Figure 3-16 Vacuolated cells.

Whole mount immunofluorescence of fixed organoids at day 23. a, CD34+ cells with multiple vacuoles and complex cytoplasm in the HLC organoid. b, cells with the same morphology are CK7+. c, CK7+ cells with multiple large vacuoles are also seen in the HLC+W/A organoid. Scale bars: 50µm, (b,c). 200µm, (a). Staining representative of 3 experiments.

3.4.15 Protocol variant mesenchymal cells and fibroblasts

PDGFRβ is expressed in cells of the HLC and HLC+W/A organoids (Fig. 17c, h), it is concentrated at the cell surface. Staining revealed no co-expression of DES and PDGFRβ (Fig. 17f, l) which together both mark HSCs. Desmin staining is localised to cells with long thin processes similar to HSCs (Fig. 17a, b, d, f, l-o) and the same as the DES+ cells of the hepatic organoid. αSMA marks the HSCs and also cells with a different morphology (Fig. 17d, m), with larger cytoplasmic areas and the appearance of myofibroblasts. These cells are less frequent than DES+/αSMA+ ones. αSMA myofibroblast shaped cells were also PDGFRβ-ve (Fig. 17g, i).

In the HLC organoid co-staining of NCAM and DES (Fig. 17a, b) revealed little or no co-expression with two separate populations of cells with similar HSC morphologies. NCAM
and αSMA are co-expressed in some cells (Fig. 17e), although separate cells only positive for each individual marker are also found with NCAM+ cells being more abundant. Cells which co-express these markers have the stretched and flattened morphology of myofibroblasts as do some αSMA+/NCAM-ve cells. These NCAM-ve cells are found in close association with αSMA-ve/NCAM+ cells and show small areas of overlapping co-expression. Some NCAM-only positive cells also have fibroblast morphology. When compared to the mesenchymal population of the hepatic organoid there appears to be an increase in NCAM+ cells in the HLC organoid. The HLC organoid also has αSMA+/DES+ dual staining cells found in the hepatic organoid (Fig. 17d).

In the HLC+W/A organoid there is more αSMA/NCAM co-expression than seen in the HLC organoid (Fig. 17j, k), these cells are further surrounded by NCAM+/SMA-ve cells and all have an activated stretched morphology. The dual positive cells appear the same as those seen in the HLC organoid, though with more and longer processes.

DES staining in the HLC+W/A organoid (Fig. 17n, o) revealed some positive cells with the same morphology as the αSMA/NCAM dual positive fibroblasts. These long stretched DES+ cells with large processes were also NCAM+ and found in areas of NCAM+/DES-ve cells, as are the NCAM+/αSMA+ cells with an activated morphology in both HLC+W/A and HLC organoids, this is different from the location of the DES+ cells in the HLC organoid with their less activated phenotype. Some individual cells express both NCAM and DES in separate areas of the cytoplasm suggesting a gain or loss of one of the markers (Fig. 17n, inset). This could be interpreted as either cells becoming activated at the onset of fibrogenesis or even inactivation after the end of a pro-fibrotic stimuli. Also seen in the HLC+W/A organoid were DES+/NCAM-ve cells with a more quiescent morphology (Fig. 17n, o) like that of the DES+ cells of the HLC organoid. This suggests there are two separate population of DES+ cells in the HLC+W/A organoid; DES+/αSMA+/NCAM+ myofibroblasts and smaller DES+/αSMA^low+/NCAM-ve cells. Figure 17o shows NCAM+ cells forming a column from the outer edge of the organoid, inwards. This is reminiscent of the mesenchymal columns of cells which give rise to HSCs in vivo (Loo and Wu 2008).
Figure 3-17 Mesenchymal and fibroblast markers in the protocol variation organoids.

Whole mount immunofluorescence of fixed organoids at day 23. a and b, Dual staining of the HLC organoid reveals separate populations of cells with a HSC morphology, thin spindle shaped cytoplasms with projections, positive for the markers NCAM and DESMIN. c and h, Cells positive for the marker PDGFRβ were seen in the HLC+W/A and HLC organoids. PDGFRβ did not co-localise with the markers DES (f, i) or αSMA (g, i). e, j, k, NCAM/αSMA dual staining shows cells that express just one marker as well as cells which co-express both, the αSMA+ cells have a myofibroblast morphology, inset shows detail. d and m, Dual immunofluorescence with DES and a different αSMA antibody shows co-expression of αSMA by all DES positive cells as well as a separate population of αSMA-only positive cells with a myofibroblast morphology in both protocol variations. n and o, NCAM staining in the HLC+W/A organoid shows co-localisation with DES, inset shows detail of co-localisation within a single cytoplasm (n). As well as NCAM+/DES-ve fibroblasts. Scale bars: 50µm, (g,i,o). 100µm, (a,b,d,f,h,j,l,m). 200µm, (c,e,k,n). Staining representative of 3 experiments.

3.4.16 Organoid seeding density affects cell viability

DRAQ5 staining on high (2-2.5x10⁶ cells) and low (1-1.25x10⁶ cells) density seeded organoids at the end of the experiment (Fig. 18a, b). In the high density experiment the
nuclear material has become diffuse with no clear boundaries, compared with the low density experiment, this suggests the cells are dead.

A comparison of the secretion of albumin into the medium, at the end of the protocol, by the organoids seeded at the different densities (Fig. 18c) shows that at high densities significantly less albumin is secreted than at low densities (a 37 fold decrease). When combined with the nuclear staining this suggests the organoids are in viable at these densities.

![Figure 3-18](image)

**Figure 3-18 Nuclear morphology and albumin staining in high and low density organoids.**

- **a** and **b**, Whole mount immunofluorescence of fixed organoids at day 23 using the nucleic acid dye DRAQ5 shows loss of nuclear integrity at high seeding densities. **c**, Albumin secretion in the medium is significantly different in the high and low seeding density organoids. n=6-10 experiments, **=p<0.01. High density= 2-2.5x10^6 cells, low density= 1-1.25x10^6 cells. Error bars represent standard error. Scale bars: 50µm.

### 3.5 Discussion

#### 3.5.1 CLCs and DLS

Cells positive for cholangiocyte markers in the organoids arranged themselves into lumen surrounding DLS. The separation of DLS and HLCs seen in the organoid (Fig. 3a) has similarities to the organisation of the portal area of the liver seen in mature bile ducts in vivo, where the mesenchymal cells surround the cholangiocytes separating them from the hepatocytes (Van Eyken et al. 1988, Coffinier et al. 2002). Although it is not apparent if the DLS of the organoid migrated into the mesenchymal cells, as in vivo, or formed there in situ. In contrast for some duct structures of the organoid the surrounding cells were positive for the fetal and cholangiocyte markers HNF1β and HNF6 (Fig. 3d, e). This difference could represent the different stages of DLS formation. In vivo cholangiocyte induction of hepatoblasts occurs around the portal vein mesenchyme leading to a ring of cholangiocytes known as the ductal plate which is surrounded on the other side by hepatoblasts (Antoniou et al. 2009). The smaller DLS in the organoid share features with
ductal plate, in contact with cells positive for hepatoblast markers, whereas the larger structures are more similar to developed ducts after migration into the mesenchyme surrounded by EPCAM-ve/HNF4α-ve cells. The different morphologies of DLS within the organoid parallels the different stages of bile duct formation seen along the hilum to periphery axis during development, where fully formed bile ducts are located at the hilum end of the liver while ductal plates is still present towards the periphery. This is only resolved after birth where bile duct morphology and markers become homogenous throughout the liver (Van Eyken et al. 1988).

The presence of CK7+/EPCAM+ cholangiocytes (Fig. 3b) allows an approximate aging of the cells in comparison to in vivo human development, with CK7+ cholangiocytes only being found after 16wpc beginning at the hilum area [Fig. 4 and (Van Eyken et al. 1988)] and EPCAM staining being lost in the ducts sometime after this point. Suggesting that the CLC and DLS are the equivalent of >16wpc fetal cholangiocytes.

HNF6 expression is seen throughout the parenchyma at early stages of liver development but is then suppressed (Landry et al. 1997, Rausa et al. 1997) except in the ductal plate where it is upregulated (Clotman et al. 2002). HNF6 expression in turn induces HNF1β expression and BEC formation (Clotman et al. 2002, Coffinier et al. 2002). In vivo its upregulation is caused by signalling from portal areas which inhibits CEBPα, itself a HNF6 suppressor (Rastegar et al. 2000, Yamasaki et al. 2006). This suggests that the CEBPα suppressing signal is found in areas of the DLS.

Interpretation of the DLS as EHBD-like is worth considering due to the way the EHBD develops in vivo, where it arises directly from the endoderm of the developing embryo (Spence et al. 2009), along with the presence of endoderm markers during the differentiation protocol (Baxter et al. 2014). The markers of the developing EHBD are almost the same as those of the IHBD, with the major differences being the IHBD is derived from CEBPα+ cells whereas EHBD is derived from PDX1+ cells also capable of differentiating into the pancreas (Yamasaki et al. 2006, Spence et al. 2009). The IHBD are morphologically different from the EHBD with smaller lumen. BECs with an EHBD morphology form in place of the IHBD in portal areas of the CEBPα KO. This is thought to be caused by a requirement of the developing IHBD to be in contact with CEBPα+ hepatocytes, which are absent in the KO. They are also absent during EHBD formation and thus maybe part of the signalling which determines IHBD specific morphology (Yamasaki et al. 2006). These differences in morphology and development of the EHBD and IHBD may also explain some of the features of the duct structures in the organoid. If clusters of cells differentiate into BECs in the organoid in areas surrounded by non-parenchymal cells
they may take on the large lumen morphology of the EHBD (Fig. 3a, b). Whereas those that form in areas surrounded by HLCs may meet the requirement for CEBPα and have the morphology of the IHBD (Fig. 3d, e).

In vivo areas of the liver containing laminin and other associated ECM proteins are sufficient to induce the formation of BEC and duct structures from bipotent progenitors and CEBPα inhibited cells (Yamasaki et al. 2006, Espanol-Suner et al. 2012). DLS structures in the organoid are shown to be in contact with laminin (Fig. 3c) and others are located at the edge of the organoids (Fig. 3d-g) where laminin is also present (Fig. 6b). This situation mimics that seen in the CEBPα KO mice where cholangiocytes and duct structures formed in capsular as well as portal regions where laminin expression is found (Yamasaki et al. 2006). Suggesting that laminin in the organoids is also associated with a BEC inducing signal and that the necessary CEBPα inhibition required to form BECs is occurring throughout the organoid, highlighting the lack of a specific portal area. This was shown in fetal liver in vitro to be caused by TGFβ treatment (Yamasaki et al. 2006). While the activin A added to the hepatic and HLC+W/A organoids could have the same effect DLS are also present in the HLC organoid which has no activin A treatment, this is indicative of organoid based TGFβ signalling.

Notch signalling from the portal mesenchyme is thought to induce biliary differentiation (Kodama et al. 2004, Tanimizu and Miyajima 2004) and culture of hepatoblasts with hepatic mesenchyme causes their differentiation into BEC (Shiojiri and Koike 1997). The abundance of mesenchymal cells (Fig. 9) in the organoid could be performing this function and hence the formation of DLS. The ability of mesenchymal cells to cause BEC differentiation can be related to the same ability seen in laminin expressing area as both are found in the portal area where duct formation occurs in vivo. Mesenchymal cells are also found in the capsular area early in development (Loo and Wu 2008, Asahina et al. 2009, Asahina et al. 2011) which forms ducts in the CEBPα KO (Yamasaki et al. 2006), these capsular mesenchymal cells further differentiate into laminin expressing MCs (Asahina et al. 2009, Asahina et al. 2011, Li et al. 2013). The cholangiocytes in the organoid then may vary on their proximity to mesenchymal and MCs leading to the different developmental stages seen in the DLS.

In the HNF6 KO duct structures are still found in portal areas, they have large lumens, disorganised laminin and delayed HNF1β expression (Clotman et al. 2002). These have similarities to some of the DLS of the organoid which are large and often disorganised. This could be caused in the animal model by the disruption of the HNF6 controlled TGFβ gradient seen during normal development which controls the hepatoblast differentiation to
BECs (Clotman et al. 2005). The lack of TGFβ gradient in the organoids may lead to the same disrupted structure and large lumens. CK staining in HNF1β LKO in postnatal mice also revealed large ducts with a disorganised and multilayered epithelium and the ductal plate was also irregular with disrupted lumens (Coffinier et al. 2002). These KO studies will not match the organoids exactly as it expresses all the missing proteins but the pathways that are disrupted in the KO may indicate where the organoid deviates from wild type development.

Cholangiocyte proliferation is controlled by the primary cilia which are osmo-, chemo- and mechano-sensitive (Masyuk et al. 2008). So any number of differences between the in vivo liver development and in vitro organoid could result in abnormal ductal proliferation such as environmental stiffness, culture medium or autocrine effects within the organoid.

During development VEGF expression is highest in the portal areas with gradients forming at the ductal plate stage (Fabris et al. 2008). VEGF has been shown to cause proliferation of cholangiocytes (Gaudio et al. 1996, Fabris et al. 2006, Gaudio et al. 2006) and may be the cause behind the large size of some of the ducts structures in the organoid which are both VEGF+ and surrounded by other VEGF+ cells (Fig. 3f). Dual KOs of FOXA1 and FOXA2 cause cholangiocyte proliferation and in turn biliary hyperplasia through increased expression of IL-6 (Li et al. 2009). This could also be the cause of CLC proliferation in the organoids, as in hepatocyte and LSEC co-culture VEGF treatment causes the LSEC to secrete IL-6 (LeCouter et al. 2003). In 3D gels of extracellular matrix epithelial cell lines can form cysts, which consist of monolayer spheres of polarised cells surrounding a central lumen. Addition of HGF to epithelial cells can induce this cyst formation and in some cases branching morphogenesis and the formation of ducts (Brinkmann et al. 1995).

Tanimizu et al (Tanimizu et al. 2007) investigated cyst formation in a liver progenitor cell line and found that both EGF and HGF can be used to increase the frequency of cyst formation and that efficiency was further increased by their use in combination. The presence of EGF in the HCM base medium in combination with the HGF added may also be responsible for the formation of the DLS in the organoid.

The differences between the CLCs in monolayer and organoid (Fig. 3) show that lumen formation and duct morphology, but not cholangiocyte differentiation itself, are dependent on 3D culture conditions and presumably the polarity and cell movement this allows. The changes in morphology of the CLCs and their organisation in the two culture formats reveal the spontaneous ability of cells in 3D systems to form structures reminiscent of those found in vivo. This is supported by the link seen between cholangiocyte differentiation and bile duct morphogenesis in development where if BEC differentiation is
disrupted so is their organisation (Clotman et al. 2002, Coffinier et al. 2002, Yamasaki et al. 2006).

3.5.2 HLCs

Polarised HLCs are found in the organoids which secrete albumin and express markers of fetal hepatocytes. The presence of the enzymes ALT and AST which function in amino acid metabolism and are used as biomarkers for hepatic injury further characterise the organoids as having hepatocyte functions.

Studies in rodent and human show liver zonation is absent during development and only occurs postnatally, its establishment is dependent on the development of acinar structure and hence the presence of portal and central veins and so would be expected to be absent from the organoids (Bennett et al. 1987, Lamers et al. 1987, Moorman et al. 1989).

In the adult human liver CPS1 is restricted to zones 1 and 2 and is not expressed in the PC zone (Moorman et al. 1989). CPS1 expression is absent in the mouse liver during development (Chen et al. 2009) but in the human liver CPS1 is expressed from 5wpc in a heterogeneous pattern in the parenchymal cells. It becomes homogeneous from 9wpc and is found throughout development (Fig. 5h), still seen in all the hepatocytes in 8 year old human liver (Moorman et al. 1989). These species specific differences highlight the importance of using human tissue as controls when possible. CPS1+/HNF4α+ HLCs found in the organoids (Fig. 5b and 13d, e) could then represent hepatocytes from 5wpc onwards while CPS1-ve/HNF4α+ cells represent early 5-9wpc hepatocyte/hepatoblasts, or adult human zone 2/3 hepatocytes.

In the adult liver GS expression is limited to the PC zone (Moorman et al. 1989) and its expression is supressed by HNF4α (Stanulovic et al. 2007). VEGF LKO studies show low levels of GS expression in non-PC regions leading to the conclusion that its expression maybe regulated by more than one pathway as HNF4α would still be found there (Walter et al. 2014). GS expression in the human liver begins after term, a postnatal marker [Fig. 5i, (Moorman et al. 1989)], showing that in respect to some markers the organoids are more mature than others. If human livers follow the rodent pattern of enzyme expression but with different timing then the liver would first be expected to become fully homogenous for CPS1 and then GS, with GS then becoming restricted to the PC hepatocytes first before CPS1 is restricted to the periportal area (Bennett et al. 1987, Lamers et al. 1987). By 8 years of age in human liver the expression of GS has become restricted to the PC zone, leaving cells which co-express GS and CPS1 in this area (and also presumably, HNF4α) (Moorman et al. 1989). This is also seen in the organoid where
the abundance of co-expression of both enzymes with HNF4α signifies largely overlapping populations of GS and CPS expression (Fig. 5b, c, and 13a-e). This pattern of expression may then indicate cells of a pre-zonation phenotype rather than an aberrant one. HNF4α+/GS-ve cells in the organoid may be equivalent to fetal hepatocytes or more mature zone 1 and 2 hepatocytes. HLCs in the organoids that are GS+ are equivalent to postnatal hepatocytes in vivo with GS/HNF4α dual positive cells being postnatal but not adult and the GS+/HNF4α-ve cells an adult PC phenotype or another cell type which expresses GS.

Lack of zonation in the organoid is revealed by the heterogeneous urea cycle enzyme expression, presumably because of the lack of clear portal or central areas showing that the presence of duct like structures is not enough to control this feature of the liver. This could be concluded from the different timing of establishment of zonation in vivo for both different areas and different enzymes within an area showing that there may not be a single control of zonation (Bennett et al. 1987, Moorman et al. 1989).

The presence or absence of WNT3a had no effect on the distribution of GS and CPS1 (Fig. 13a, b, d, e) despite the role of WNT in vivo, where its activity has been shown to convert PC gene expressing hepatocytes into periportal expressing ones (Colletti et al. 2009) and so might have been expected to increase GS expression at the expense of CPS1. The lack of response to WNT agrees with the earlier conclusion that these cells are pre-zonal due to either a lack of maturation or acinar structure.

OSM and DEX are added to in vitro cultures of mouse fetal hepatocytes to induce maturation (Kinoshita et al. 1999, Kamiya et al. 2001) the effects of which on the HLCs can be examined by comparison of the hepatic organoid with the HLC and HLC+W/A organoids. This showed no change in GS/HNF4α co-expression or the overlapping expression areas of GS and CPS1 revealed by HNF4α co-expression (Fig. 13a-e) showing no sign of maturation. Suggesting that some effects of OSM and DEX treatment are species specific, as they were used in mouse hepatocytes where they also induce CPS1 expression (Chen et al. 2009) but this is not necessary in the hepatic organoid nor would it be a sign of human hepatocyte maturation. Or alternatively showing that OSM and DEX, or analogues, are already present in the organoid and/or maturation does not affect zonation.

HNF6 and HNF1β are expressed in the hepatic organoid (Fig. 5e, f), in vivo they act as markers of bipotential hepatoblasts and immature hepatocytes (Yamasaki et al. 2006). During development HNF1β is expressed in the liver diverticulum (Barbacci et al. 1999, Coffinier et al. 1999) before being increased in the ductal plate and decreased in the
parenchyma (Clotman et al. 2002). HNF1β and HNF4α co-expressing cells in the organoid (Fig. 5e) may represent the ductal plate like cells regressing to form hepatocytes (Coffinier et al. 2002) or earlier parenchymal cells. With HNF1β-ve cells representing the more mature HLCs which have lost it expression. The HNF1β+/HNF4α-ve cells appear to cluster together perhaps showing areas of duct formation. As CEBPα suppresses HNF6 in vivo (Rastegar et al. 2000) the HNF6 and CEBPα co-expression in the organoid (Fig. 5f) suggests the cells are differentiating to either form CLCs or more mature HLCs, depending on whether CEBPα expression is going up or down in the individual cells.

MRP2 is found on the apical side of hepatocyte membranes where it marks the bile canaliculi (Mayer et al. 1995, Tanaka et al. 1999). It also indicates hepatocyte polarity and its expression is lost as they dedifferentiate in vitro (Rowe et al. 2013). MRP2 mediates ATP dependant transport of glutathione conjugates, xenobiotics and organic anions from the hepatocytes to the canaliculi (Keppler et al. 1997) and so plays an important role in drug metabolism (Gerk and Vore 2002). MRP2 expression in the HNF4α+ cells of the organoid (Fig. 5d) shows the potential of the HLCs for examining drug metabolism and its arrangement into canaliculi shaped structures shows the hepatocytes have acquired polarity as in vivo.

The albumin gene is regulated by glucocorticoid in rats in vivo and DEX upregulates albumin in hepatocytes in vitro (Nawa et al. 1986). In vitro cultures of fetal hepatocytes require both DEX and OSM to maintain albumin production for longer than 5 days, the effect was dose dependent up to 10ng/ml OSM and 10⁻⁷M DEX (Kamiya et al. 1999); the concentrations used in the HLC and HLC+W/A organoids. Albumin production in the hepatic organoids after 23 days in culture along with the lack of significant difference after OSM and DEX treatment in the HLC and HLC+W/A organoids (Fig. 13f) suggests that these factors either have no effect or similar ones are already present. Cortisol is present in the base medium (HCM) used to differentiate the organoids and it, like DEX, binds the glucocorticoid receptor. The presence of hematopoietic cells in the organoids, of which CD68+ monocyte/macrophages are a derivative, may also suggest the presence of OSM which is expressed by the hematopoietic cells of the fetal liver (Kamiya et al. 1999).

TGFβ has also been shown to maintain albumin levels in rodent fetal hepatocytes but this was only over a 2-3 day period (Sanchez et al. 1995, Kamiya et al. 1999). While the hepatic organoid is activin A (a member of the TGFβ superfamily) treated this ends on D12 of the protocol, 11 days before the end of protocol ALB assay and so seems unlikely to be the reason for sustained ALB production. This is reinforced by the HLC protocol having no activin A treatment but not significantly different ALB levels. OSM and DEX
treatment however continues until the end of the protocol (D16-23) and so is comparable to the previous in vitro work.

3.5.3 Vacuolated cells

CD34+ individual cells seen in the HLC organoids (Fig. 16a) maybe endothelial precursors, or angioblasts, like those found in the portal areas during/prior to capillary formation (Nakanuma et al. 1997, Gouysse et al. 2002). This would explain their appearance in only the HLC organoid which has a different endothelial-like cell population than the other protocols. Alternatively CD34 also marks hematopoietic cells which occur in the liver during development.

CK7 staining in HLC and HLC+W/A organoids also revealed heavily vacuolated CK7+ cells (Fig. 16b, c). They were more numerous in the HLC organoid suggesting that the addition of WNT3a and activin A inhibits there formation. If these are the same population of cells as the CD34+ ones they may be equivalent to the CK7+/CD34+ cells reported to have been isolated from fetal liver (Lemmer et al. 1998, Suskind and Muench 2004). These cells were shown to be large with a high cytoplasmic complexity, in vitro culture led to the conclusion that they were HSC precursors

3.5.4 Endothelial and LSEC marker expression in the organoids

Formation of vasculature from EC precursors is termed vasculogenesis (Risau and Lemmon 1988). These precursors, termed angioblasts, are proliferative and migratory (Patel-Hett and D'Amore 2011). FGF2 in vivo has a role in induction of angioblasts from mesoderm (Cox and Poole 2000) and the same may occur in the organoid which is FGF2 treated. If the organoids match in vivo development vasculogenesis would occur in mesoderm derived cells at an early stage leaving small CD34+/CD31+ structures, followed by angiogenesis to extend the structures.

The capillaries of the STM are formed by vasculogenesis and then undergo further angiogenesis upon invasion of the STM by the hepatic endoderm (Couvelard et al. 1996, Collardeau-Frachon and Scoazec 2008) to give rise to the sinusoids of the liver. These then acquire LSEC specific markers during development such as discontinuous cell junctions (DeLeve et al. 2004) and LYVE1 expression (Mouta Carreira et al. 2001). LSEC CD34 expression is reduced during development beginning at 15-25wpc until it is absent in the adult liver (Gouysse et al. 2002). CD31 is initially found in the sinusoids as they develop from the STM (Asahina et al. 2011) after which it is lost by 10wpc (Gouysse et al. 2002)
before being expressed again from 25wpc (Lalor et al. 2006) continuing in the adult (Pusztaszeri et al. 2006). The same process of continuous endothelium gaining LSEC specific features seems to occur in the organoid with the heterogeneous co-localisation of either CD31 or LYVE1 with CD34 (Fig. 7c-f) showing a change from capillary-like to liver specific ECs, as the acquisition of LYVE1 can be used as a marker of tissue specific EC differentiation from pre-existing ECs or progenitors (Nonaka et al. 2007). At E14.5 in mice FACS analysis has shown liver ECs that co-express CD31/CD34/LYVE1 (Nonaka et al. 2007) which match the cells seen in the organoids (Fig. 7f). This marker expression is equivalent to roughly 10wpc in humans (Enzan et al. 1997, Nonaka et al. 2007).

It has been stated that markers of continuous ECs and the laminin matrix are both undetectable in the sinusoids after 10wpc with LSEC specific features detectable after 15wpc (Gouysse et al. 2002). The unexpected co-expression of the continuous endothelial marker CD34 and the sinusoid specific LYVE1 seen in the 18wpc FL (Fig. 7i) may be due to changes in the ECM in the areas surrounding the larger vasculature. Sinusoids have a specific minimal basement matrix (Hahn et al. 1980) which they acquire during their differentiation from unspecialised capillaries. At this point in development it lacks laminin whereas the central vein maintains its classic endothelial ECM including laminin and CD34 expression which marks continuous endothelium and fits with the CD34+ structure in Figure 7i. While CD34 is not expected to mark the portal vein or artery at this time (Gouysse et al. 2002). This change in ECM between the vasculature structures could result in localised capillarization of the adjacent LSECs. Alternatively it could be caused by paracrine signalling of the ECs or a VEGF gradient as different amounts of VEGF expression specify different EC phenotypes (more VEGF more sinusoidal) (Walter et al. 2014) with the developing biliary ducts generating high amounts of VEGF in portal areas (Fabris et al. 2008).

A de-differentiation of LSECs is seen in vivo during aging and disease, known as capillarisation (Le Couteur et al. 2001). This is associated with a reduction in fenestrae and scavenger behaviour, changes in the basement membrane and an increase in CD31 expression (DeLeve et al. 2004). In vitro cultured LSECs require a source of VEGF to prevent this capillarisation (Yokomori et al. 2003, DeLeve et al. 2004). Walter et al found capillarisation in the LSECs of VEGF reduced mice with increased CD31 and laminin (Walter et al. 2014). Conversely it can be hypothesised that an increase in VEGF signalling leads to a decrease in CD31 and laminin. Heterogeneous CD31 staining in organoids (Fig. 7c, d, f, and 15d) suggests there is a source of VEGF but that it is insufficient to prevent
capillarization or has not yet had time to specify an LSEC phenotype from pre-existing capillaries.

Taken together the in vivo and in vitro data suggest a continuous scale for capillarisation is possible within individual endothelial structures; with CD31+/CD34+ cells and a continuous endothelium at one extreme, as seen in capillaries in vivo and areas co-expressing these markers in the organoid; followed by CD34+/LYVE1+/- endothelium; and finally LYVE1+/CD31-ve/CD34-ve LSECs.

CD31+/LYVE1-ve vasculature structures in the organoids could have the phenotype of the PBP a network of portal capillaries which express markers of continuous endothelium (Gouysse et al. 2002). Which would mean the addition of WNT3a and activin A intended to induce portal signalling to allow biliary duct formation instead induced a portal phenotype on the ECs.

Filopodia are seen extending from some of the cells positive for EC markers in the organoids (Fig. 7b, f, and 15d). ECs with filopodia are described as tip cells. Functionally specialized tip cells and their filopodia are a sign of active angiogenesis. Non-proliferative they are the leading edge of growing endothelial structures, with proliferation occurring in adjacent cells known as stalk cells (Gerhardt et al. 2003). Tip cells are involved in anastomosis (Phng et al. 2013) and their presence in the organoid is suggestive of VEGF signalling as in vivo they migrate along a VEGF gradient towards areas of high expression (Gerhardt et al. 2003). The remaining cells of the organoid endothelial structures, which lack filopodia, would then constitute stalk cells which in vivo are specified by Notch mediated lateral inhibition from the tip cells, they are both proliferative and lumen forming (Gerhardt et al. 2003, De Smet et al. 2009). Figure 7b shows the presence of both filopodia and merging cellular vesicles, a sign of lumen formation (Kamei et al. 2006), in different areas of the structure matching the description of tip and stalk cells.

The growth of these structures could be induced by WNT signalling which causes proliferation in human ECs (Masckauchan et al. 2005) and in LSEC in an autocrine fashion via β-catenin in vivo (Klein et al. 2008). WNT3a in the culture medium may take that role in the organoid as it too is capable of signalling via β-catenin.

Comparison of the HLC+W/A and HLC organoids (Fig. 15a, b) shows the addition of WNT3a and activin A during the differentiation induces the formation of the branching endothelial structures that are CD34+. These structures are also seen in the WNT3a and activin A treated hepatic organoids (Fig. 7a-f).

In vivo LYVE1 is acquired by pre-existing vasculature as opposed to the formation of structure by LYVE1+ cells (Nonaka et al. 2007). This does not appear to be the case with
the LYVE1+ cells in the HLC organoid (Fig. 15b) which lack structure and show no sign of lumen formation. However LSECs are thought to acquire their tissue specific phenotype during vasculogenesis, prior to LYVE1 expression, in the form of STAB expression (Nonaka et al. 2007). Leading to the hypothesis that in the HLC organoid the lack of WNT and activin A leaves EC progenitors which can still acquire a hepatic identity becoming disconnected LSECs, while the same progenitors in the other protocols are induced to form structures prior to LYVE1 expression.

Endothelial WNT signalling maintains vascular stability in vivo during sprouting angiogenesis, WNT3a has been shown to be capable of this function in human umbilical vein endothelial cells (HUVEC) ex vivo (Phng et al. 2009). This role of WNT in stabilising endothelial structures may explain the lack of structure yet presence of ECs seen in the HLC organoid. These cells untreated with WNT3a, in contrast to those of the other two protocols, may not be able to maintain endothelial structures.

Crosstalk between WNT and VEGF pathways may provide further mechanism for the lack of endothelial structure in the HLC organoid. A decrease in WNT signalling in LSECs causes a decrease in their VEGFR expression (Klein et al. 2008). Which in turn may reduce their response to VEGF which drives vasculogenesis (Morell et al. 2013) and is found in the organoids potentially secreted by the HLCs and CLCs (Fig. 3f). The requirement of WNT signalling for LSECs is met with the hepatic and HLC+W/A organoids where its presence may increase the effect of the secreted VEGF by increasing VEGFR expression and promoting vasculogenesis.

The relative absence of LYVE1+ cells in the HLC monolayer compare to the organoid (Fig. 15b, c) shows that the 3D culture system induces the proliferation of ECs or their precursors.

The differences between hepatic organoid and monolayer (Fig. 3a-g) show that 3D cultures allow the development of structures which mimic those found in vivo but also changes the markers expressed by the individual cells. Revealing it affects how the cells differentiate as well as how they form structure suggesting that the specification of a correct phenotype is dependent upon the cells polarity. Also suggesting that the inverse maybe true and the removal of structure from primary cells by plating in monolayer maybe the cause of their dedifferentiation, as seen in ECs which lose their tissue specific phenotypes during in vitro culture (Daneker et al. 1998, DeLeve et al. 2004). Here the organoids could act a source for these cells allowing the maintenance of their tissue specific phenotype from which they could then be isolated when required. More generally organoids could act as a source for
cell types which cannot be differentiated without 3D culture induced polarity, co-culture or both.

3.5.5 Macrophages marker expression in the organoids

Kupffer cells are a liver specific macrophage that reside in the sinusoids in close association with the LSECs with which they act as a powerful scavenger system removing waste products from the blood supply (Elvevold et al. 2004). They are a hematopoietic cell and have a different mesodermal origin than that of the mesenchymal liver cells, they are not STM derived (Asahina et al. 2009, Asahina et al. 2011). Hematopoiesis begins in the liver around 5wpc and continues until at least 20-24wpc. This overlaps in the beginning with yolk sac hematopoiesis, until 8-10wpc, and then with bone marrow hematopoiesis from 11wpc (Kelemen et al. 1979). The liver produces macrophages during development visible during the 5-16wpc period. CD68+ macrophages are found in the liver from 5wpc (Slayton et al. 1998). Macrophages are phagocytes (Gordon et al. 1992) which differentiate from monocytes (Gordon 1986). CD68 is localised to the lysosome and endosomes of macrophages and monocytes (Saito et al. 1991), it shares homology with membrane shuttling proteins (Fukuda et al. 1988), which shuttle vesicles between lysosomes, endosomes and the plasma membrane (Lippincott-Schwartz and Fambrough 1987). The CD68 staining in cells in the hepatic organoid (Figure. 8a) is located in round subcellular compartments which lack the cytoplasmic DRAQ5 staining seen in the rest of the cell which indicates its localisation to specific subcellular structures, potentially lysosomes or endosomes. The CD68+ cells of the organoid also co-express CD31 a marker found on Kupffer cells and monocytes in vivo (McMichael 1987, Lalor et al. 2006).

Monocytes and macrophages are hematopoietic cells derived from the CD45+ cells which act as the progenitors for all red and white blood cells. Their presence in the organoid suggests other hematopoietic cell types may also be present. Production of hematopoietic cells and macrophages are both found within the developing liver and so maybe expected if the organoid is successfully mimicking in vivo liver development.

An alternative explanation for the LYVE1+/CD34-ve cells of the HLC organoid (Fig. 15b) is that they too represent macrophages, subsets of which may be LYVE1+ in vivo (Schledzewski et al. 2006). This could fit with the abundance of CD34+ potentially hematopoietic cells found in the HLC organoid, as macrophages would be derived from these cells. Though the LYVE1+ cells have a different morphology from the CD68+ cells of the hepatic organoid. This would also imply that the addition of WNT3a and activin A is
responsible for inducing not just vasculature structure but the differentiation of all the ECs, which would then be entirely absent from the HLC organoids.

3.5.6 **Mesenchymal and mesothelial marker expression**

All the variations of marker expression in the mesenchymal and mesothelial cells of the organoids can be accounted for by the different cell types and stages seen in development. In vivo the mesothelial lining is derived from the STM and has been shown to give rise to subMCs, HSC and other liver mesenchymal cell types throughout development hence there shared markers of DES and NCAM (Loo and Wu 2008, Asahina et al. 2009, Asahina et al. 2011). MCs gain an epithelial cell type during development first with the gain of expression of epithelial associated cytokeratins and then ECAD by 20wpc. The presence of analogues of STM derivatives in the organoids, such as MCs and HSCs, suggests a recapitulation of STM-like cells and its role in development as well as the derivation of hepatic stellate-like cells from MCs as described in vivo (Asahina et al. 2009, Asahina et al. 2011).

Laminin is found in vivo in the ECM of the developing liver as part of Glisson’s capsule (Amenta and Harrison 1997), expressed by the MCs (Li et al. 2013), and in portal areas in contact with the bile ducts (Carpentier et al. 2011, Espanol-Suner et al. 2012). This shared expression between the outer capsule and portal mesenchyme is expected if the portal areas are considered invaginations of Glisson’s capsule, also referred to as a Glissonian sheath. Which may also explain the shared ability of portal and capsule areas to induce BECs for CEBPα KO hepatocytes (Yamasaki et al. 2006). The surrounding of the organoids by a laminin layer (Fig. 6b) suggests the presence of functional MCs.

Hepatic organoids have a CK7+ outer layer of cells with mesothelial morphology, absent from HLC and HLC+W/A organoids (Fig. 6a and 14a, c). Cells of the HLC+W/A organoid, which shares WNT3a and activin A treatment with the hepatic organoid, did however have the long thin morphology of MCs which may be equivalent to the CK7-ve/CK19+ MCs found at earlier stages of liver development (Fig. 14a). This is reinforced by the presence of cells of a mesothelial location and morphology in the HLC+W/A organoid which co-express both DES and NCAM (Fig. 14b) consistent with an early liver MC in vivo; which express NCAM until 8wpc, DES from 7-20wpc (Loo and Wu 2008) and CK7 after 8wpc (Fig. 6e). This would suggest that OSM and DEX treatment slow or inhibits the maturation of the organoid MCs, while WNT3a and activin A induces it.
DES is expressed by a small number of activated HSCs in human adult liver (Burt 1999) but by most HSCs in the fetal liver (Loo and Wu 2008), suggesting that the DES+ cells in the organoid are fetal as they do not have the morphology of activated myofibroblasts.

DES marks the cells of the STM before invasion of the foregut and its derivatives afterwards which include fetal HSCs, subMCs and PMCs (Loo and Wu 2008, Asahina et al. 2009, Asahina et al. 2011). SubMCs were αSMA-ve in human development (Loo and Wu 2008) suggesting that the dual DES+/SMA+ cells of the organoid were not subMCs. Whereas fetal HSCs in human (Loo and Wu 2008) and mice, and PMCs in mice (Asahina et al. 2009) did co-express these markers. In vivo PMCs are defined by their proximity to the veins, which makes HSC-like a better description of these cells as the organoids lack veinous structures. In vivo fetal HSCs were reported as αSMA+ from different time points by different groups (Schmitt-Graff et al. 1991, Enzan et al. 1997, Cassiman et al. 2002), with 15wpc (Loo and Wu 2008) being the earliest. At this time point in vivo most HSCs co-expressed DES, though some cells were αSMA+/DES-ve, and began to lose their NCAM expression (Loo and Wu 2008) all of which also describes the mesenchymal cells of the organoid suggesting that they represent a HSC-like population at a later than 15wpc time point.

SubMCs of the human liver are lost at 11wpc they are NCAM+/DES+/αSMA-ve (Loo and Wu 2008) this shows that the organoid cells are not subMCs and if the HSCs are derived from them, as in development, it further suggests the mesenchymal cells of the organoid are more mature than 11wpc. SubMC are cuboidal shaped and transition into spindle shaped cells, the latter of which better describes the morphology of the DES+ cells seen in the organoid, where no cuboidal cells are seen.

In vivo from 15wpc the HSCs became αSMA+ (Loo and Wu 2008), and NCAM staining becomes progressively weaker, NCAM-ve stellates have been reported at 16wpc (Lee et al. 2007), and αSMA marked a larger HSC population than NCAM or DES (Loo and Wu 2008) showing a heterogeneous population of HSCs in vivo which is also seen in the organoid. By 18-20wpc NCAM+ HSCs were restricted to sinusoids near the mesothelium and portal tracts (Loo and Wu 2008). The lack of NCAM co-expressing cells (Fig. 9a) suggests the organoid DES+ cells are at an age equivalent to, and potentially over, 20wpc. The αSMA staining of the organoid also marks a population of cells which includes all the DES+ ones as a subset (Fig. 9c), there could then be present three distinct HSC populations; DES+/αSMA+, DES-ve/αSMA+ and DES-ve/NCAM+ potentially with separate origins.
In the adult rat NCAM is expressed in HSCs but no other fibroblast or SMC populations (Knittel et al. 1996). Suggesting the NCAM+/DES-ve cells could represent adult HSCs. The αSMA+/DES-ve cells in the organoids have a fibroblast morphology (Fig. 9c). In vivo the liver has several populations of αSMA+ myofibroblasts, some are derived from the activated HSCs during fibrosis, and others are found associated with veins and arteries (Cassiman et al. 2002).

In vivo in adult mice DES/PDGFRβ co-expression is found in most HSCs, although cells expressing only one marker are also seen (Henderson et al. 2013). However no co-expression of PDGFRβ with DES was seen in the organoids (Fig. 9e). The PDGFRβ+ cells (fig. 9f) could represent a population of more mature HSCs which in adult humans are DES-ve/αSMA-ve (Schmitt-Graff et al. 1991). HSC are a type of pericyte (Lee et al. 2007) and an alternative interpretation of the PDGFRβ+ cells of the hepatic organoid could be as non liver-specific pericytes. Pericytes surround ECs in vivo, as the PDGFRβ+ cells surround the CD34+ cells in the organoid (Fig. 9f). They are involved in both EC development (Hirschi et al. 1998, Hellstrom et al. 1999, Hirschi et al. 1999) and function (Lindahl et al. 1997) where they stabilise blood vessels, their loss leads to vessel regression (Hammes et al. 2002).

In the hepatic organoid NCAM and DES mark separate populations but DES and αSMA are co-expressed, except for a small number of myofibroblasts, this along with the absence of NCAM+ fibroblasts means αSMA and NCAM would mark mostly separate populations in the hepatic organoid. Which is in contrast to the αSMA and NCAM dual staining of the HLC and HLC+W/A organoids which show αSMA+/NCAM+ cells. Although lost from the HSCs during development NCAM is highly expressed by aHSCs in adult human fibrotic and cirrhotic livers (Cassiman et al. 2002). This could indicate that the αSMA+/NCAM+ cells of the HLC and HLC+W/A organoids are activated HSCs. In diseased livers NCAM staining is negative in the SMC and portal and septal myofibroblasts, ruling out these populations (Knittel et al. 1996).

The HLC+W/A organoid contains the highest number of fibroblast cells with the most extreme activated morphology, longest and most numerous processes, as well as the strongest NCAM+/DES+ co-expression and NCAM+/SMA+ co-expression. This could be due to the treatment of these cells with both activin A and OSM. OSM is a pro-inflammatory cytokine related to IL-6, which are thought to be responsible for HSC activation in vivo (Friedman 2008). Activin A is part of the TGFβ superfamily it is structurally similar and signals through SMADs; as does TGFβ a known inducer of HSC activation (Gressner et al. 2002).
The major HSC like population in the hepatic organoid was thus found to be DES+/αSMA+/NCAM-ve/PDGFRβ-ve consistent with fetal HSCs. DES+ spindle shaped cells are present throughout all variations. In the HLC and hepatic organoids all the DES+ cells share a smaller quiescent phenotype. OSM and DEX containing protocols had an apparent increase in NCAM+ fibroblast morphology cells and sequential activin A and OSM treatment resulted in large amounts of cells gaining activated morphology and markers in the HLC+W/A organoid.

3.5.7 DAPM treated hepatic organoids

The BEC of the rat liver, in the common bile duct and IHBD, are injured by DAPM exposure leading to their necrosis and shedding into the lumen (Kanz et al. 1992, Kanz et al. 1995). This is thought to be caused by exposure to DAPM metabolites excreted into the bile, as bile from DAPM treated rats is sufficient to cause BEC damage when infused into the common bile duct of untreated rats (Kanz et al. 1995). Excretion into bile is a common process by which xenobiotics are passed out of the liver following their conjugation in phase II reactions (Klaassen and Watkins 1984) particularly for glutathione conjugates (Vore 1993). Cholangiocytes express GGT (Manson 1983) which may act on glutathione conjugates of DAPM, the most prominent DAPM metabolite in the bile of rats, cleaving terminal glutamate residues (Chen et al. 2008). It was also shown that higher levels of glutathione protect against DAPM toxicity as glutathione depletion in hepatocytes followed by low (50mg/kg) DAPM treatment resulted in noticeable hepatocyte damage including apoptotic cells and increases in bile acids and phase II enzyme activity. As well as increasing the rate of BEC injury (Kanz et al. 2003). Thus glutathione is important for the detoxification of DAPM metabolites (Chen et al. 2008).

The biliary system shows damage before hepatocyte injury, with little change in serum LFTs at minimal doses (Kanz et al. 1998). Specifically no change in ALT or GGT was detected at 25 or 50mg/kg DAPM in rats, a dose which causes marked BEC damage with up to 50% of the BEC surrounding lumens being exfoliated while minimal hepatocyte injury was observed (Bailie et al. 1993, Kanz et al. 1998, Kanz et al. 2003). BEC began to detach from the basement membrane as soon as 6 hours after DAPM treatment (Kanz et al. 2003), with maximum biliary injury seen at 24hr, (Limaye et al. 2010). Thus DAPM is proposed to be a selective bile duct toxicant at minimally toxic doses (Kanz et al. 1998).

Rats treated with DAPM at a high dose of 250mg/kg showed elevated ALT and alkaline phosphatase (ALP). This high dose resulted in more rapid and severe injury with exfoliation and loss of BEC from the ducts (Kanz et al. 1992, Kanz et al. 1995). While
100 mg/kg doses resulted in necrosis of periportal hepatocytes (Bailie et al. 1993). Showing its effects on both the hepatocytes and cholangiocytes at higher doses.

Metabolic activation of DAPM is CYP450 mediated (Kajbaf et al. 1992, Bailie et al. 1993), involving oxidation to N-hydroxylamine and nitrosoarene (Kajbaf et al. 1992), highly reactive compounds (Eyer 1988) which are then thought to be detoxified by conjugation. Bailie et al propose DAPM requires bioactivation to cause toxicity. This is carried out by a CYP450 that can be inhibited by Aminobenzotriazole (ABT) which was shown to ameliorate DAPMs toxic effects (Bailie et al. 1993). ABT has its greatest inhibitory effect on CYP3A4 and CYP2A6 with their activity essentially eliminated in human liver microsomes (Linder et al. 2009), both of which were shown to be expressed by monolayer HLCs using a similar protocol (Baxter et al. 2014).

Together with the organoid results these data suggest HLCs metabolise and bioactivate DAPM in a CYP450 dependent manner which then acts on the CLCs of the DLS. At low doses this results in damage to the CLCs and their structures but not HLCs, acting as a cholangiotoxin (Fig. 11), while at high doses it is toxic to both CLCs and HLCs as shown by the increased AST and GGT levels (Fig. 10i, j), the loss of HNF4α expression and disruption to CK+ cell morphology and DLS (Fig. 10g). These different effects at high and low doses mimic those seen in vivo.

The presence of analogues of both canaliculi (Fig. 5d) and bile ducts (Fig. 3a-g) in the organoid, which in vivo are connected, shows the potential for recapitulation of interactions between hepatocytes and cholangiocytes that involve the in vivo architecture of the liver. Further suggesting the possibility that in the organoid as in vivo the reactive metabolites of DAPM are secreted into the canaliculi by HLCs before being drained into the DLS. The canaliculi are labelled by MRP2 a membrane protein capable of transporting potentially detoxified DAPM glutathione conjugates (Keppler et al. 1997), while GGT expressed by BECs and shown to be present by its release in the damaged organoids, can cleave these conjugates potentially rendering them toxic again and explaining the specific site of damage.

Also notable was the continued expression of CD34 by cells of the organoid along with no apparent disruption of the organisation of these cells into structures (Fig. 10h) at doses shown to affect the CLCs and HLCs; further highlighting DAPM specificity.

Transdifferentiation from hepatocytes to cholangiocytes has been shown to occur in toxicity models where normal cholangiocyte self-renewal is impaired. Studies in rat liver have shown that when BEC are injured by DAPM their response to bile duct ligation (BDL) is altered. Instead of the usual proliferation of cholangiocytes (Polimeno et al.
there is a transdifferentiation of hepatocytes to cholangiocytes (Michalopoulos et al. 2005). After DAPM treatment periportal hepatocytes were found to begin expressing the biliary markers OV6 and CK7. Chimeric rats were used to trace the hepatocytes after DAPM treatment leading to the hypothesis that these hepatobiliary cells repair the duct structures and are transdifferentiating hepatocytes acting as facultative stem cells for cholangiocytes (Michalopoulos et al. 2005).

The formation of hepatobiliary cells and repair of ducts by transdifferentiating hepatocytes does not require BDL. Chronic biliary liver disease (CBLD) in humans involves progressive BEC degeneration and the formation of hepatobiliary cells (Desmet et al. 1995, Chen et al. 2006, Limaye et al. 2008). Limaye et al used repeated DAPM treatment to mimic CBLD (Limaye et al. 2010). The use of hybrid rats to trace hepatocytes again showed BEC derived from them. Both DAPM-BDL and repeated DAPM treatment resulted in HNF4α/CK19 co-expressing ducts interpreted as undergoing regenerating, as well as periportal hepatocytes expressing CK19. HNF1β and HNF4α co-expressing ductal cells were also seen in areas of ductular reaction (Limaye et al. 2010).

HNF4α+/CK7+ hepatobiliary cells in the organoid were also located in proximity to DLS which is analogous to in vivo where the transdifferentiating cells are periportal hepatocytes. It suggests proximity to the DLS structures rather than just DAPM treatment is necessary for their formation which may be indicative of signalling from the damaged DLS to the hepatocytes. Also seen are individual round CK7+ cells which maybe damaged CLCs. These also fit the description of oval cells however oval cell formation is inhibited by DAPM treatment (Petersen et al. 1997) suggesting that the co-expression of hepatobiliary markers seen in the organoid is not a liver stem cell/progenitor response.

After DAPM and BDL treatment some hepatocytes can be seen forming a ductular structure (Michalopoulos et al. 2005) also seen in the lumen surrounding HNF4a+ cells in the organoid (Fig. 11d). Similarly hepatocytes arranging themselves into lumen surrounding structures are seen in mice fed a diethoxycarbonyl dihydrocollidine (DDC) diet. Where the cells co-expressed the hepatic and biliary markers HNF4a and SOX9 and can be further differentiated into CK19+ duct cells (Tanimizu et al. 2014). Showing hepatocyte transdifferentiation is a response common to multiple models of cholangiocyte injury.

The duct structures in parenchymal areas of the CEBPα KO have hepatobiliary features, HNF4α and CK19 expressing cell surrounding lumen (Yamasaki et al. 2006), and are reminiscent of the second bilayer seen transiently in ductal plate formation where HNF4α cells also surround lumen (Antoniou et al. 2009). A TGFβ gradient concentrated on the
portal area during development plays a role in BEC differentiation from hepatoblasts (Clotman et al. 2005), and CEBPα KO has been shown to have the same effect as TGFβ treatment on fetal liver in vitro (Yamasaki et al. 2006). This TGFβ induced formation of a transient hepatobiliary phenotype is seen again after DAPM treatment where increased TGFβ1 is seen in periportal areas (Limaye et al. 2010), as well as a CK+ ductal plate like ring around the portal vein and HNF4α+ cells surrounding lumen also expressing CK (Michalopoulos et al. 2005). The results described all occur in a potentially similar situation with originally HNF4α expressing cells responding to BEC inducing signals. Together this suggests that the transdifferentiation response is similar to the formation of ducts during development and that the organoids respond to DAPM treatment in the same way.

The organoids could be used to examine this phenomenon in human cell based systems. The absence of this marker co-expression in the untreated organoid and the subsequent ability to induce it shows the fidelity of the organoids in comparison to in vivo liver, and highlight another potential use for them. Not only do they die correctly but they also recapitulate the “exotic” regenerative features seen in specific injuries. Collectively this data shows that CK7+ cells not only organise themselves like cholangiocytes but are also functionally similar in their response to damage. It also shows the secretion of enzymes from the organoid which mimic the response shown by damaged livers in vivo. The organoids show the same threshold response in respect to high and low doses of DAPM as seen in vivo resulting in general and cholangiocyte specific damage respectively. The HNF4α+/CK7+ cells suggest the organoids are capable of repair and transdifferentiation both features of the liver in vivo and could be used to further investigate this process.

### 3.5.8 Organoid seeding density

Comparison of the DE cells seeding density of the organoid shows that at high densities (2-2.5x10^6) cells appear dead/damaged with the use of a nuclear dye and also secrete significantly less albumin into the medium. The high densities may cause necrosis of the cells by preventing the diffusion of oxygen and nutrients.

### 3.5.9 Interactions between cell types in vitro

The presence of GATA4+ non-endoderm cells in the protocol (Baxter et al. 2014) seems to mimic in vivo development, where GATA4 is expressed by the mesoderm surrounding the
developing liver bud (Si-Tayeb et al. 2010). KO experiments prove this is crucial for liver bud expansion (Watt et al. 2007). It is also known to regulate BMP signalling in vivo which is necessary for hepatic specification of the endoderm derived cells (Nemer and Nemer 2003). BMP2 is added to the culture medium to differentiate the organoids but the presence of BMP secreting and regulating cells within the organoid potentially providing the equivalent role they do in vivo may result in both a more appropriate dose and improved temporal and spatial distribution. Such interactions could happen between all the cell types within the organoid that are analogous to those of the developing liver in vivo, making up for inadequacies or simplifications within the protocol. The closer the match to the conditions of liver development in vivo the organoid culture in vitro is the more autonomous its development maybe, leading to a more liver-like organoid.

Further examples of the interdependencies between cell types in liver development include; the expression of HGF by the HSC (Bennett et al. 1987) and expression of both HGF and pleiotrophin by MCs and subMCs from the fetal liver (Asahina et al. 2009). Co-culture with human liver fibroblasts supports the culture of hepatocytes (Jodon de Villeroche and Brouty-Boye 2008). Endothelial cells even before they become functional in the circulatory system have been shown to promote the growth, morphogenesis and differentiation of hepatic progenitors (Matsumoto et al. 2001, Lammert et al. 2003, Matsumoto et al. 2008). In hepatocyte and LSEC co-culture VEGF treatment causes the LSEC to secrete HGF and IL-6 resulting in hepatocyte proliferation (LeCouter et al. 2003). The combined presence of these cell types may improve HLC differentiation, and CLCs via IL-6, even without a complex differentiation medium.

Pericytes via PDGFβ play a role in the formation of vascular structure (Hellstrom et al. 1999, Semela et al. 2008), as does VEGF. Different reports show VEGF expression from different liver cell types. It has been found in the early human liver parenchyma (Gouysse et al. 2002) and is involved in the development of both bile ducts and the portal capillaries (Fabris et al. 2008). VEGF expression is found in proximity to the DLS in the organoid.

The inclusion of multiple cell types makes the differentiation more alike in vivo development requiring less complexity at the protocol design end. The in vitro interactions described are seen in two cell type containing systems, the scale of the complexity and interactions must be much greater in a multiple cell type system. All of which suggest that co-culture could provide a more complete and complex culture environment than that found in single cell populations.

The potential benefits of co-culture extend beyond the development of the organoid. Non-parenchymal cells play important roles in liver disease. In BDL the PDGF signalling based
interaction between the bile ducts and hepatic stellate cells drives ductular reaction/cholangiocyte proliferation and HSC activation leading to fibrogenesis (Grappone et al. 1999, Kinnman et al. 2000, Kinnman et al. 2001). To study this response in vitro would require co-culture of cholangiocytes and HSCs and the organisation of the cholangiocytes in biliary structures; an example of a potential use for liver organoids. Use of human cells instead of mice may better mimic human disease and response providing an advantage over animal models.

3.5.10 Cell polarity and the formation of structure

Epithelial cells which line structures are orientated so that the sides facing lumen are the apical surface, lateral surfaces contact other cells and basal surfaces attach to the ECM. In vitro contact between the substrate and cells occurs at the basolateral membrane in cultured epithelial cells which is also the membrane in contact with the blood supply in vivo. Thus 2D culture of cells prevents the development of correct polarity which can cause dedifferentiation (Zegers et al. 2003). Monolayer culture is also obviously unsuitable for the formation of 3D structures.

Many of the cells of the organoid show markers of polarity as well as formation into structures analogous to those found within the liver. These include: the formation of square CLCs surrounding lumen, like cholangiocytes in biliary ducts; MRP2 expression in channels adjacent to HNF4α+ cells, like the canaliculi of hepatocytes; an outer layer of cells positive for MC markers and ECM proteins, like Glisson’s capsule; branching structures of cells positive for endothelial markers with filopodia, signs of lumen formation and surrounding mesenchymal cells like the LSECs, capillaries and pericytes. These structures are all absent from the monolayer experiments using the same protocol showing they develop spontaneously in the organoid culture system.

The development of structure is important as in vivo the function and phenotype of cells is intimately linked to their structure as seen by their loss in dedifferentiating primary cells in monolayer culture, in hepatocytes (Rowe et al. 2013) and LSECs (DeLeve et al. 2004, Lalor et al. 2006). Its importance is also seen during development, for example in cholangiocytes where their differentiation and morphogenesis is linked, which has implications for the differentiation of cells in monolayer. Highlighted by the difference in morphology seen between the CLCs in monolayer and organoid culture (Fig. 3) and by the difference in marker expression using the same comparison in the endothelial-like cells (Fig. 7a-g). The presence of structure was again important in the DAPM assay in assessing the damage done to the DLS and also potentially in the mechanism of action itself.
3.5.11 Summary

Directed differentiation of ESCs as organoids resulted in cells with the characteristics of hepatocytes, cholangiocytes, mesothelial cells, endothelial cells, HSCs, fibroblasts and macrophages all of which are found within the liver in vivo.

The hepatic organoids contained endoderm derived hepatic cell types as well as liver appropriate stromal, endothelial and hematopoietic cells which in vivo have a mesoderm origin, all from a single starting population. GATA4+ cells mark lateral mesoderm in development and later the STM. All the mesoderm cells seen in the organoid can trace their origin to these structures during in vivo development. That the protocol based on liver development works on mesoderm cells as well as endoderm cells is not surprising considering they are in the same environment in vivo.

The presence of multiple cell types may allow the formation of more detailed models allowing development to occur in an autonomous manner making up for short comings in protocol design. It also allows the study of effects that involve multiple cell types, which is arguably most if not all assays of liver function or injury/insult in vivo, and the interactions between them. They could also potentially be of use in the future as a source of transplant material in a way that a homogenous population of hepatocytes are not.

The cells spontaneously formed structures reminiscent of liver architecture including; DLS, vasculature structures, canaliculi-like channels and a mesothelial and ECM containing outer layer. This shows that the formation of their requisite structure is inherent to the differentiation of the corresponding cell types when grown in co-culture in a 3D format.

The formation of vasculature structure was found to require the addition of WNT3a and or activin A while for the formation of DLS its addition was unnecessary. And despite the wide use of such for in vitro culture of hepatocytes and their differentiation from PSCs, the addition of OSM and DEX had no effect on albumin secretion.

Mimicking in vivo development in the organoid resulted in fetal-like cells as seen by comparison to staining of primary fetal tissue and in the literature; this matches our previous findings despite the differences in protocol and culture conditions showing that these cells lack an adult maturation signal. The only obvious exception being GS expression, a postnatal marker, which may have been induced by the in vitro environment.

The hepatic organoids showed a multicellular response to DAPM toxicity and signs of regeneration of structure as determined by changes in morphology and LFTs which are the
standard clinical measure of hepatic damage. This matches the response seen in vivo, and hints at interactions between cell types showing the organoids can be used to model toxicity.

An appropriate next step for experimentation with the organoids could involve their in vivo transplant into immune compromised mice to assess their ability to anastomose to the circulatory system, and if successful their ability to form ectopic livers. Also of interest is the ability of the mesenchymal cell types of the organoid to mimic the in vivo fibrotic response of HSCs. This could be further combined with investigation of whether the cells positive for macrophage markers can simulate inflammation which in vivo can drive liver fibrosis.
4 General Summary

The differentiation of HLCs in monolayer and hepatic organoids with characteristics of hepatocytes and livers found in vivo shows that mimicking in vivo development by addition of soluble factors is a viable strategy.

Both organoid and monolayer protocols produced cells which more closely resemble their fetal rather than adult counterparts. This may suggest that the cells are lacking a maturation signal. The fetal phenotype of differentiated HLCs impacts upon their ability to be used for drug metabolism which uses proteins expressed in higher quantities in adult cells, making their maturation an important research area. The use of the thyroid hormone T3 which is involved in human development throughout the embryo is one potential candidate currently being investigated in our lab for its ability to induce maturation. Alternatively a developmentally immature phenotype may be a feature inherent to using ESCs due to residual expression of ESC gene regulatory networks in differentiated cells, as determined by comparison of pre-existing datasets covering the differentiation of different cell types from ESCs (Cahan et al. 2014).

The presence of multiple cell types in the organoid suggests they are also present within monolayer differentiation and by extension other unsorted differentiation protocols. It also shows the potential benefits of multiple cell type containing systems for use in toxicity modelling. As the DAPM results seen would not have been possible by assay on a single cell type.

Human hepatocytes can be transplanted from cadavers (Fisher and Strom 2006), which suggests that successfully differentiated ES cells could also be used as a source of hepatocytes for human transplantation. Diseases such as inborn errors of metabolism could also be treated by iPSC derived and corrected HLCs (Fisher and Strom 2006, Grompe 2006). HLCs could also be used to improve mouse models, fah deficient mice can have up to 90% of their hepatocytes replaced by human hepatocytes (Azuma et al. 2007, Bissig et al. 2007). Such mice could narrow the gap between human and animal models of development disease and toxicity allowing some of the features of human specific hepatocytes to be studied. Another strategy allowing human livers to be grown in other large organisms has the added advantage of scale allowing a potential production line of hepatocytes using chimeric pigs as incubators to improve upon PSC differentiation protocols (Rashid et al. 2014). Fully realised organoids derived from ESCs have the potential to supplant all of the above models as well as being used for transplants allowing an indefatigable source of livers overcoming the major hurdle of donor organ availability.

Less complete models could also have a role in bioartificial liver devices (Strain and
Neuberger 2002), which would aim to temporarily provide basic liver functions for people awaiting transplant or to provide their own liver a chance to regenerate.
5 Supplementary methods and results

5.1 Supplementary Methods

5.1.1 Immunoblotting

5.1.1.1 Protein isolation

Cells were rinsed in PBS (Sigma Aldrich, UK) then harvested in RIPA buffer (Sigma Aldrich, UK) with Protease Inhibitor Cocktails I and II (Fisher Scientific, UK) by scraping cells from the tissue culture plastic, the cell suspension was transferred to an eppendorf tube. It was homogenised by pipetting and centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernatant was transferred to a new eppendorf tube and the concentration quantified using the Bio-Rad Bradford based protein assay (Bio-Rad Laboratories, UK). Samples were then diluted in RIPA and 5X Laemmli buffer [320mM Tris (pH 6.8), 5% SDS, 25% Glycerol (Fisher Scientific Ltd, UK), 0.5% β-mercaptoethanol (Sigma Aldrich, UK) diluted in dH2O].

5.1.1.2 Protein electrophoresis by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Laemmli buffer, in a mini-PROTEAN Tetra Electrophoresis system (Bio-Rad Laboratories, UK), assembled according to manufactures instruction.

Stacking gels were made using 125μM Tris (pH 6.8), 4% Acryl/bis 29:1 40% (w/v) solution (Amresco, USA), 25μl 20% SDS, 12.5μl TEMED (Sigma Aldrich, UK), 150μl 10% APS (Sigma Aldrich, UK) diluted to 5 ml with dH2O. Resolving gels were made of 26mM Tris (pH 8.8), 10% acrylamide, 0.1% SDS, 12.5μl TEMED, 300μl of 10% APS diluted to 10ml with dH2O. Resolving gel was poured and then overlaid with isopropanol and allowed to set. Isopropanol was then poured off and rinsed with dH2O. The stacking gel was then poured onto the resolving gel and Teflon well combs inserted. The gel then set at room temperature.

Protein samples diluted in RIPA and Laemmli buffer were heated to 100°C for 5 minutes and vortexed. 20mg of protein was loaded per well of the 10% acrylamide gel, along with a protein ladder (7-175kDa ColorPlus Prestained Protein marker, New England Biolabs, UK). The gel was submerged in Tris-glycine running buffer (25mM Tris base, 200mM Glycine, 100ml Methanol diluted to 1 litre with dH2O) and proteins were separated by running a current of 200V through the gel for ~1 hour, until the dye front ran out.
5.1.1.3 Transfer of protein to a nitrocellulose membrane

The gel was sandwiched with a Hybond ECL membrane (Scientific Laboratory Supplies, UK) between blotting paper, with a sponge on either side. The sandwich was constructed under water to reduce air bubbles in the system, and was enclosed in a transfer cassette, placed into the tank and covered with Tris-glycine transfer buffer (250mM Tris base, 2M Glycine, 35mM SDS diluted to 1 litre with dH₂O). The proteins from the gel were transferred to a nitrocellulose membrane by running a current of 100V through the transfer tank for 90 minutes. Once complete the membrane was removed from the transfer cassette and stained with Ponceau Red (Sigma Aldrich, UK) to check for a successful transfer.

5.1.1.4 Protein detection

The membrane was blocked with 5% milk in PBS with 1% Tween (Sigma Aldrich, UK) for an hour, then incubated with the primary antibody (Supplementary Table 2-1) diluted in 5% milk/ PBS/1% Tween and put on a rocker overnight at 4°C. The membrane was next washed 3x for 5 minutes, with 1% Tween in PBS and incubated with a HRP-conjugated secondary antibody diluted in 5% milk/PBS/1% Tween for 1 hour at room temperature on a rocker. The membrane was washed 3x for 5 minutes with 1% Tween in PBS. Signal was detected using either ECL Advance Western Blotting Detection kit (GE Healthcare, UK), SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) or UptiLight HS WB HRP Chemiluminescent Substrate (Interchim, France). The membrane was covered with the appropriate chemiluminescent reagent for 5 minutes in the dark. Signal was detected using a ChemiDoc automated detection system (Biorad Universal hood 2, Bio-Rad Laboratories, UK) and the Quantity One (version 4.6.7.) software. After images had been captured the membrane was washed 3x for 5 minutes with 1% Tween in PBS and then incubated with Monoclonal HRP-linked anti β-actin-peroxidase (1:50000, Sigma Aldrich, UK) for 30 minutes at room temperature with agitation by rocking. The membrane was washed 3x for 5 minutes with 1% Tween in PBS; the signal was detected using the same system as before.

5.1.2 Generation of protein heat map and iTRAQ analysis

“Tissue or cell homogenates, prepared in triethylammonium bicarbonate buffer/0.1% sodium dodecyl sulphate (TEAB/SDS) by sonication, were clarified by centrifugation...
(10,000g, 2 minutes) and supernatant protein concentration determined by Bradford assay. 8-plex iTRAQ reagent labelling (Applied Biosystems) was carried out according to the manufacturer's instructions using 100μg protein for each sample. Sample complexity was reduced by strong cation exchange and fractions of 2 mL were collected. Liquid chromatography (LC) followed by tandem mass spectrometry (MS/MS) of peptide-rich fractions was performed on a QSTAR Pulsar I hybrid mass spectrometer (AB Sciec). In addition, each run included a common reference preparation consisting of pooled samples from the different experiments. Quantitation of proteins was relative to this common pooled sample. Proteins identified by two or more peptides with at least 90% confidence, or by a single peptide with at least 99% confidence, were included in subsequent analyses. Protein quantities, relative to the common reference pool, were used as input when comparing proteomes. The relationships between samples were inspected as a heatmap using Euclidean distance for proteins common to all samples.” From (Rowe et al. 2013).

5.1.3 Isolation of RNA

5.1.3.1 Harvesting RNA

RNA was prepared using the RNeasy Mini Kit (Qiagen, UK). Cells were harvested by aspirating the culture medium and washing the cells with PBS. 350μl Buffer RLT (Qiagen, UK) with 1% β-mercaptoethanol (Sigma Aldrich, UK) to the cells which were then harvested with a cell scraper; β-mercaptoethanol is added to buffer RLT to denature RNases released upon cell lysis. The cell suspension was transferred into an RNase free eppendorf and homogenised by pipetting. RNA was purified using spin columns from the RNeasy Mini Kit (Qiagen, UK). Ethanol was added to the cell suspension in a 1:1 ratio to aid subsequent RNA binding to the silica membrane within the spin column. The cell suspension was then transferred to the RNeasy spin column placed in a 2ml collection tube and centrifuged for 15 seconds at 10,000rpm, which causes binding of total RNA to the silica membrane within the spin column. The flow through was discarded from the collection tube. The RNA bound to the silica membrane underwent a series of washes at 10,000rpm using buffer RPE and buffer RW1. The final wash was discarded and the empty spin column placed in a 2ml collection tube then centrifuged for 1 minute at 10,000rpm to ensure the flow through was fully discarded from the spin column. The RNA was eluted by adding 30μl RNase-free water to the RNeasy spin column placed in a 1.5ml collection tube and centrifuged for 1 minute at 10,000 rpm.
5.1.3.2 DNase treatment of RNA

DNase treatment to denature genomic DNA was carried out to prevent its subsequent amplification. Total RNA was DNase treated using a DNase kit (Sigma Aldrich, UK). All samples and reagents were kept on ice. The RNA was DNase treated as follows; 30μl of RNA, 1.75μl of DNase I and 3.5μl of 10X buffer was incubated at room temperature for 15 minutes. To stop the reaction 3.5μl of stop solution was added to each sample and incubated at 70°C in a water bath for ten minutes.

5.1.3.3 RNA quantification

2μl of RNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, USA). RNA concentration was detected by absorbance readings at 260nm. To check RNA purity the absorbance at 260nm was compared to the absorbance at 280nm, a ratio of ~2 (260nm/280nm) confirmed the sample was free from contamination by phenol, alcohol and protein.

5.1.4 Cell culture

All Stem cells and MEFs were cultured as described in 3.5.2.2 except for the H-line specific differences described below.

5.1.4.1 H-line specific medium and passage

H-line medium contained; 200ml DMEM-F12, 50ml KOSR, 2.5ml NEAA, 2.5ml L-glutamine BME solution (5ml L-glutamine and 3.5μl 2-mercaptoethanol), 25μl FGF2.

To passage H1/H9 cells medium was first aspirated from the culture. Collagenase (collagenase type IV 1mg/ml in DMEM F/12, Gibco) was added to cover the cells, 1ml/well for a 6-well plate, which were then incubated for 6 min at 37°C. hES cells were observed under a microscope, and left until the edges of the colonies started to dissociate and the feeders started to pull away. The collagenase was aspirated then the cells washed with 1ml of medium which was also aspirated. Then medium was used to detach the cells from the flask. The cell suspension was transferred to fresh feeders at the desired ratio.

5.1.4.2 HLC differentiation

To differentiate to HLCs, 70% confluent monolayers hES cells in 6-well plate format were grown in a series of different culture mediums; 2 days in 3ml/day of stage 1a medium;
RPMI, Activin A at 100ng/ml (Peprotech), WNT3A at 25ng/ml (R&D systems), 0.5% FBS, penicillin/streptomycin. 2 days in 3ml/day of Stage 1b medium; RPMI, Activin A, 0.5% FBS, penicillin/streptomycin. 6 days in 2ml/day Stage 2 medium; HCM (Lonza), BMP2 at 20ng/ml (Peprotech), FGF2 at 30ng/ml. 5 days in 2ml/day Stage 3a medium; HCM, HGF at 20ng/ml (Peprotech). 15 days in 2ml/day Stage 3b medium; HCM, oncostatin M at 10ng/ml (Sigma Aldrich, UK), dexamethasone at 100nm. Medium was changed daily during the experiment.

5.1.4.3 HEPG2 culture

HEPG2 were grown in a monolayer at 5% CO2 and 37 °C in complete medium (Dulbecco's modified Eagle's medium + L-glutamine containing antibiotics supplemented with 10% fetal bovine serum) (PAA Laboratories).

5.1.4.4 Fetal and adult hepatocyte culture

Human fetal and adult hepatocytes were cultured in Williams E medium supplemented with; ITS 1%, L-glutamine 1%, Pen/strep 1%, dexamethasone 100nM and incubated at 5% CO2 and 37°C. Cells were seeded at a density of 1.5 million cells per well onto Matrigel coated 6-well plates. These were overlaid 3 hours later with a second layer of Matrigel. Media was exchanged every 2 days.

Human adult hepatocytes were sourced commercially from Invitrogen (part of Life Technologies) which obtained surgical resections from living donors as part of a clinical organ donation scheme. As such, the organs had been clinically assessed as free from liver disease. Following organ retrieval, cells were prepared overnight in the Invitrogen facility in Warrington, UK. As soon as commercial quality assurance measures had been obtained on post-isolation cell viability by trypan blue exclusion, hepatocytes were transported in suspension in proprietary iced transport medium. Transport never lasted more than 1h. On arrival in the laboratory, cells were immediately centrifuged and resuspended twice in cell culture medium and an aliquot taken for cell counting. Viability of an aliquot was determined by trypan blue exclusion and was >90% for each donor. High attachment efficiency was observed in conventional Matrigel culture with very few cells evident in the media aspirated after 3h when the Matrigel overlay was applied. Human fetal samples were collected immediately upon medical or surgical termination of pregnancy by our research nurses and delivered to the research laboratory within 1h. Immediate dissection isolated the liver which was either fixed for immunohistochemistry or processed for protein isolation or
cell culture. The latter was achieved by repeated passage of the tissue gently through the
tip of a 1ml pipette until an even cell suspension was acquired. Adapted from (Rowe et al.
2013).

5.1.5 Biochemical assays

5.1.5.1 Urea assay
Urea secretion was quantified using a QuantiChrom Urea Assay Kit (Universal
Biologicals). Equal amounts of reagent A and B were allowed to equilibrate at room
temperature and then combined. 5μl of sample was added in duplicate per well of a clear
bottomed 96 well plate. To this was added 200μl per well of working reagent. The plate
was left at room temperature for 20 mins after which optical density was read at 520nm.
Duplicates of a blank and standard were also included on the plate and urea concentration
was calculated using the equation;
\[
[Urea] = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} \times n \times [STD] \text{ (mg/dL)}
\]

5.1.5.2 CYP3A assay
CYP3A activity was assayed using P450-Glo™ CYP3A4 Assay Luciferin-PFBE
(Promega). Reconstitution buffer was added to the lyophilized Luciferin Detection Reagent
and homogenized. 2mM Luciferin PFBE was diluted 1:40 in stage 3B medium and 1ml
was added to each well of differentiated cells to be assayed. Cells were incubated with
luciferin PFBE for 6 hr after which the medium was collected. 50μl of sample was added
to a single well of a white opaque 96 well plate, in duplicate. To each well was added
200μl of reconstituted luciferin detection reagent, the plate was then left at room
temperature in the dark for 20 mins. Luminescence was then recorded using a luminometer
and relative light units were compared to a standard curve derived from known quantities
of luciferin.

5.1.5.3 ADH activity
Samples were pipetted and sonicated to homogenize in 50mM pH 8.8 (Sigma Aldrich, UK)
sodium pyrophosphate buffer. Then centrifuged at 14,000rpm for 1 minute. The reduction
of nicotinamide adenine dinucleotide (NAD) to NADH during the catalysis of ethanol to
acetaldehyde as indicated by increase in absorbance at 340nM was assayed by spectrophotometry over 5 minutes.
6 References


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