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NOVEL HUMAN *IN VITRO* SYSTEMS FOR STUDIES OF DRUG INDUCED HEPATOTOXICITY

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Stockholm 2012

Published by Karolinska Institutet. Printed by [name of printer]

Cover artwork courtesy of Ina Schuppe-Koistinen

ISBN 978-91-7457-869-0

ABSTRACT

Drug-induced liver injury (DILI) is a major human health concern, estimated to account for about half of all cases of acute liver failure in the general population. DILI also represents a significant problem in drug discovery, being one of the most common reasons for regulatory actions, including boxed warnings, restricted marketing and withdrawals of marketed drugs. Adverse drug effects in the liver are hard to detect at an early stage during drug development, much owing to the shortcomings of the currently available pre-clinical model systems. The work presented in this thesis aimed to refine and further develop more sensitive, human *in vitro* models and methods for better prediction of DILI and the underlying mechanisms.

Mono-culture of human primary hepatocytes is the closest *in vitro* model to the human liver, currently considered the golden standard in drug development. However, limitations, such as low availability of qualitative liver tissue and phenotypical instability of these cells in culture, require new sources of functional human hepatic cells. In this thesis, we have shown that high-density culture of the human hepatoma cell line Huh7 induces a spontaneous, hepatic differentiation process, without the need for inducers as is usually the case. A particular increase of *CYP3A4* gene- and protein expression and catalytically activity was observed. Moreover, we found that the large increase in CYP3A4 expression seen in the confluent Huh7 cells is mediated by PXR nuclear translocation and increased PXR mediated transcriptional activity, most likely as a result of decreased CDK2 activity and cell cycle arrest. The high constitutive expression of CYP3A4 in the confluent Huh7 cells makes this cell system useful for studies of mechanisms for regulation of PXR and the *CYP3A4* gene.

The unique characteristics of stem cells make them an attractive large-scale source of hepatic cells for drug development and safety assessment. Using a novel stepwise differentiation protocol we have been able to differentiate human embryonic stem cells (hESC), via definitive endoderm and progenitor stages to hepatocyte-like cells which exhibit many hepatocyte-specific features and functions, including CYP metabolic activities. A dynamic three-dimensional (3D) bioreactor system was shown to prolong and maintain the specific functions of primary hepatocytes, as well as facilitate the hepatic maturation of hESC into hepatocyte-like cells.

It has become increasingly evident that inflammatory event plays a significant role in many DILI events. Thus, *in vitro* systems containing a population of immune competent cells in combination with hepatic cells could be of great significance for studying mechanisms underlying DILI. A co-culture cell model consisting of hepatocytes and monocytes has been developed where the cells were separated by a semipermeable membrane. The hepatotoxic drug troglitazone caused a potentiated and more rapid cytotoxic effect in cells treated in the co-culture compared to the single cultures. Troglitazone treatment also resulted in an increased expression of several stress-related genes in the co-cultures compared to the single cultures. These results suggest a synergistic cytotoxic effect by soluble mediators released by the cells and underscores the importance of incorporating several different hepatic cell types in order to generate more sensitive *in vitro* systems and better prediction of DILI.

LIST OF PUBLICATIONS

I. Edling Y*****, **Sivertsson L***, Butura A, Ingelman-Sundberg M and Ek M (2009). Increased sensitivity for troglitazone-induced cytotoxicity using a human *in vitro* co-culture model. *Toxicol In Vitro,* 23:1387-1395.

II. Brolén G, **Sivertsson L**, Bjorquist P, Eriksson G, Ek M, Semb H, Johansson I, Andersson TB, Ingelman-Sundberg M and Heins N (2010). Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage. *J Biotechnol,* 145**:**284-294.

III. **Sivertsson L**, Ek M, Darnell M, Edebert I, Ingelman-Sundberg M and Neve E.P.A (2010). CYP3A4 catalytic activity is induced in confluent Huh7 hepatoma cells. *Drug Metab Dispos,* 38:995-1002.

IV. **Sivertsson L***, Synnergren J*****, Jensen J, Björquist P, Ingelman-Sundberg M. Hepatic differentiation and maturation of human embryonic stem cells cultured in a perfused three-dimensional bioreactor. *Stem Cells Dev, 2012 Sep 12. [Epub ahead of print]*.

V. **Sivertsson L**, Edebert I, Porsmyr Palmertz M, Ingelman-Sundberg M and Neve E.P.A. CDK2 mediated PXR activation induces CYP3A4 expression in confluent Huh7 hepatoma cells. *Submitted.*

***** Contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
A1AT	Alpha-1antitrypsin
ADR	Adverse drug reaction
AFP	Alpha-fetoprotein
ALB	Albumin
BMP	Bone morphogenetic protein
CAR	Constitutive androstane receptor
CDK	Cyclin-dependent kinase
CK	Cytokeratin
CLEM4	Constitutive liver enhancer module
CXCL	Chemokine (C-X-C motif) ligand
CYP	Cytochrome P450
DDIT3	DNA damage-inducible transcript 3
DE	Definitive endoderm
DILI	Drug induced liver injury
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
FGF	Fibroblast growth factor
FOXA	Forkhead box protein
hESC	Human embryonic stem cells
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigens
HNF	Hepatocyte nuclear factor
IL	Interleukin
iPSC	Induced pluripotent stem cells
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MT2A	Metallothionein 2A
NK	Natural killer
OCT	Octamer-binding transcription factor
PKA/PKC	Protein kinase A/ Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PROX	Proximal promoter region
PXR	Pregnane X receptor
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
RXR	Retinoid X receptor
siRNA	Small interfering RNA, short interfering RNA or silencing RNA
SOX	SRY (sex determining region Y)-box
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UGT	UDP-glucuronosyltransferase
XREM	Xenobiotic-responsive enhancer module

1 INTRODUCTION

Thanks to extensive research, new and improved drugs are constantly being developed with the intention to prevent, treat and cure diseases. Several important discoveries in pharmacology, such as the antibiotics, have improved our physical and mental wellbeing and have markedly increased the average life expectancy of humans (about 70%) during the last century [1]. When properly used, medical drugs are generally safe and effective for most patients, however, adverse drug reactions (ADRs) do occur and may be lethal for the patient. During the later years the number of reported ADRs in patients has increased drastically, uncorrelated with the prescription level of drugs [2]. A Swedish study showed that ADRs are responsible for more than 10% of all hospital admissions [3]. Moreover, ADRs cause about 3% of all deaths in the general population which makes it the seventh most common cause of death in Sweden [4]. Similar numbers has been presented for other European countries [5, 6], as well as for USA [7]. Apart from individual suffering, ADRs constitute a considerable economic burden for the community, valued to cost almost as much the drug treatment itself [3, 8].

In recent years the pharmaceutical industry has been struggling with low rate of new drug candidates, long discovery processes and increasing developmental costs. Discovery and marketing of a new drug typically takes 10-15 years and costs around 900 million USD [1]. Thus, ADRs also have a major economic impact on the pharmaceutical industry, resulting in unsatisfactory marketing approval rates, post-marketing restrictions, boxed warnings and withdrawals of marketed drugs [9, 10]. Due to insufficient pre-clinical methods the industry also fights with high failure rates during later stages of drug development, many of them caused by toxicology and clinical safety issues [1]. The failure rates in phase III clinical trials are estimated to exceed 40% with hundreds of million dollars lost for the drug companies [11].

One of the most commonly reported idiosyncratic drug reactions are liver related injuries [12, 13]. Idiosyncratic drug reactions are hard to detect during pre-clinical studies since the toxic reactions are unpredictable based on what is known about the pharmacological properties of the drug. Moreover, idiosyncratic drug reactions often occur after some period of latency [13]. Drug induced liver injuries (DILI) are serious events which may cause acute liver failure, leading to liver transplantation or even death of the patient. In order to decrease the above mentioned problems there is an urgent need to improve the biological significance of the pre-clinical models. To date, the principle source of information regarding possible liver related ADRs is based on laboratory animal testing (*in vivo* methods) in combination with various cell culture models (*in vitro* models). Due to inter-species differences, *in vivo* studies have low predictive value for drug related toxic effect in humans. In addition, the use of animals raises major ethical and societal concern [14, 15]. According to existing animal protection EU legislation (Directive 86/609/EEC), pharmaceutical companies are obliged to apply available methods to replace, reduce and refine (“The 3R’s”, Russel and Burch, 1959) the use of animals in both safety and efficacy evaluations. Thus, innovated, sensitive and reliable humanized *in vitro* models that better mimics the *in*

vivo situation in the liver are essential for an effective and accurate preclinical evaluation of new chemical entities and have been addressed in the thesis presented here.

1.1 THE HUMAN LIVER

1.1.1 Embryonic liver development

Life begins with the fertilized egg (zygote), the combined, haploid set of chromosomes from two different individuals (Figure 1). The dividing zygote grows into a blastocyst with a flattened cavity called the epiblast. By a morphogenetic process named gastrulation some cells from the epiblast are rearranged by an inward movement, through a structure called the primitive streak, and the three germ layers are formed; ectoderm, endoderm and mesoderm, which gives rise to the different organs and tissues in the body [16]. The liver is developed from the endoderm germ layer which is suggested to arise from the mesendoderm, a common precursor cell population to the mesoderm [17]. The TGFβ/Activin/Nodal signaling factor group, belonging to the transforming growth factor beta (TGFβ) superfamily, together with Wnt signaling has shown to be particular important for initiation of gastrulation and definitive endoderm formation [16, 18, 19]. Within the endodermal linage high Nodal signaling activates different nuclear transcription factors, such as FOXA2 (forkhead box protein, also called HNF-3β), SOX17 (SRY (sex determining region Y)-box 17) and GATA4 [20-22]. These transcription factors, in turn, activates and regulates the transformation of the definitive endoderm into a two dimensional sheet of cells, ultimately forming the primitive gut tube [17, 23, 24]. The gut tube becomes regionalized and the hepatic progenitor cells of the ventral foregut endoderm are stimulated to form the liver bud. This process is stimulated by the interaction with the surrounding mesoderm tissues [25] secreting fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) [20, 26, 27]. The specialized hepatic progenitor cells in the growing liver bud are called hepatoblasts and express liver specific markers, such as alpha-fetoprotein (AFP), albumin (ALB) and fetal forms of cytochrome P450s (CYPs), as well as biliary epithelium markers like cytokeratin19 (CK19) [28]. Thus, the hepatoblasts has shown to be bipotential, capable of differentiating into both hepatocytes and cholangiocytes [29]. The organogenesis is regulated by the interplay and flucturation of different growth factor released from surrounding tissues [20, 30] but also by infiltrating hematopoietic cells that influence liver maturation by secretion of cytokines, like Oncostatin M (OSM) and tumor necrosis factor α (TNFα) [31, 32]. The transcription factor HNF4α (hepatocyte nuclear factor 4α) is not only a vital player for early embryonic development and hepatocyte maturation, but also for metabolic regulation and proper liver function in the adult liver [33]. The fetal liver differs from the adult liver regarding specific functions such as CYP activities, exhibiting a change in metabolic profile after birth to be able to cope with the exposure to endotoxins and xenobiotics [34].

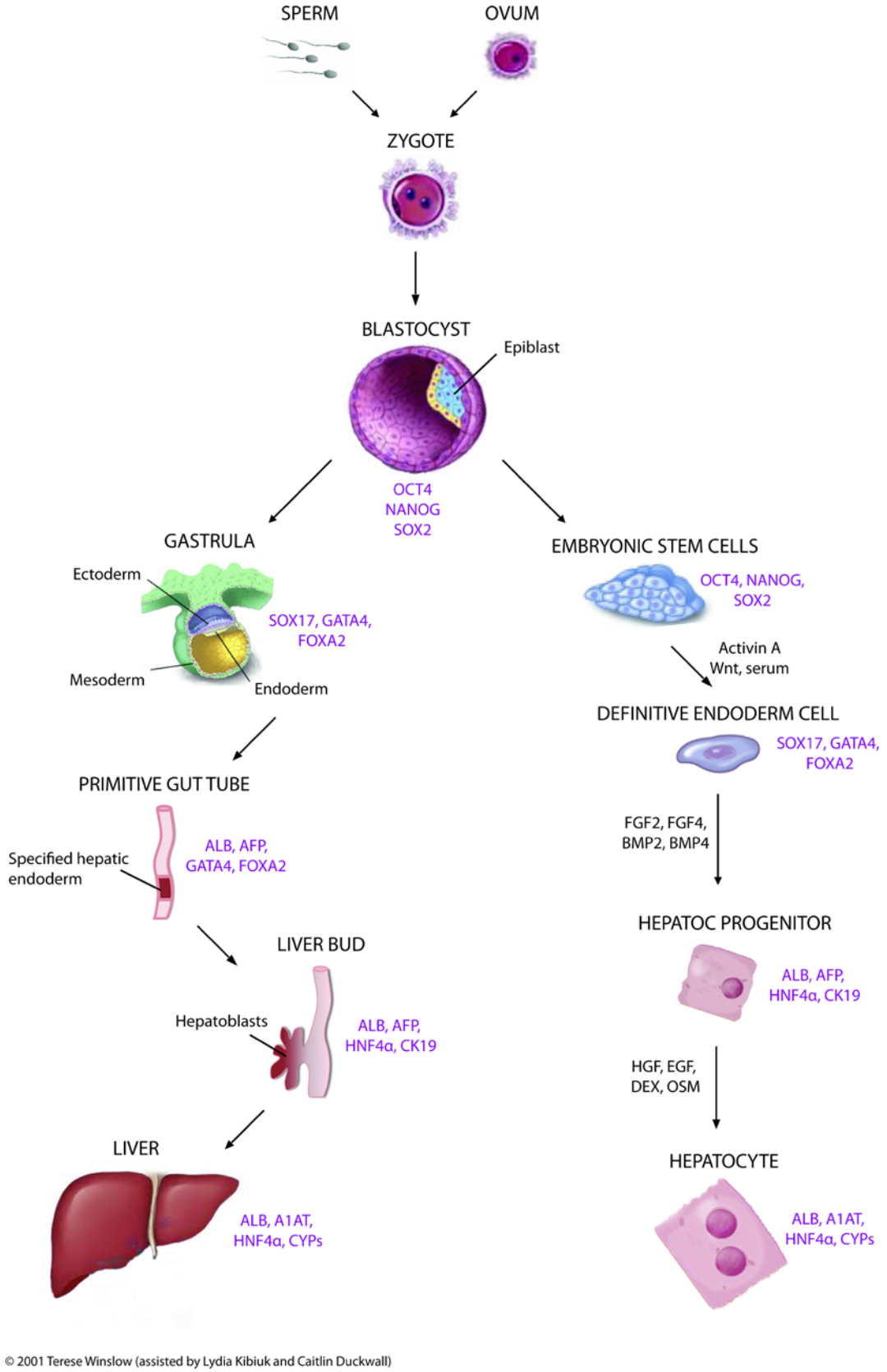

Figure 1. Schematic illustration of embryonic liver development compared to hepatic differentiation of human embryonic stem cells (hESC). Genes expressed at different developmental/differentiation stages are shown in pink. Selected soluble factors, typically added during hepatic differentiation of hESC, are also shown. Illustrations adapted from [35] with permission from Terese Winslow. OCT4, Octamer-binding transcription factor; SOX2, SRY (sex determining region Y)-box 2; A1AT, Alpha-1antitrypsin.

1.1.2 Liver structure and functions

The liver is the largest internal organ in the body and performs several essential functions. These include bile production, plasma protein synthesis, glucose homeostasis and glycogen storage, processing and storage of fats, such as cholesterol, and production of hormones [36]. The liver is a highly specialized tissue that comprises many different cell types, further described below. From a histologically perspective the liver consists of small functional units called hepatic lobules. The liver is supplied with oxygenated blood from the hepatic artery and venous blood from the portal vein entering the periportal area of the lobule and via branches of small interlobular vessels. The mixed blood flows through vascular channels called sinusoids and leaves the lobule via the hepatic central vein located in the center of the lobule. Bile is secreted by the hepatocytes into bile canaliculi, flows in the opposite direction of the blood and empties into the bile ducts that are lined by epithelial cells called cholangiocytes. The bile is ultimately secreted into the duodenum where it facilitates the digestion of lipids [36]. The lobule is divided in zones based on functionality. The concentration of oxygen, nutrients, insulin and glucagon is highest in the periportal area and decreases towards the central vein. As a result of the concentration gradient, hepatocytes in the different zones have different morphology and function [37]. For example, hepatocytes around the central vein have higher density of endoplasmic reticulum and possess the highest levels of enzymes involved in detoxification and bioinformation [38, 39]. Substances from orally consumed food and drugs reach the liver via the venous blood from the intestine, which is filtered through the liver before entering the systemic blood circulation. This makes the liver a central organ in metabolism of both endogenous substances, such as bilirubin and ammonia, as well as exogenous substances, like bacterial toxins and alcohol [36]. Most pharmaceutical drugs available on the market today are administered orally which makes the liver a highly exposed organ for drug toxicity. Due to its central position in the body, the liver also functions as an important immune organ harboring many cells involved in both the innate and the adaptive immune response [40].

1.1.3 Hepatic cell types

The liver is comprised of several different cell types, all with unique and vital functions (Figure 2). The predominant cell type, the parenchymal cells, is the hepatocytes which constitute about 70% of all the hepatic cells. Of the non-parenchymal cells, the sinusoidal endothelial cells comprise the major part, followed by immune cells (Kupffer cells and lymphocytes), biliary cells (cholangiocytes) and stellate cells [40, 41]. The liver also harbors small amounts of liver specific stem cells called oval cells [42].

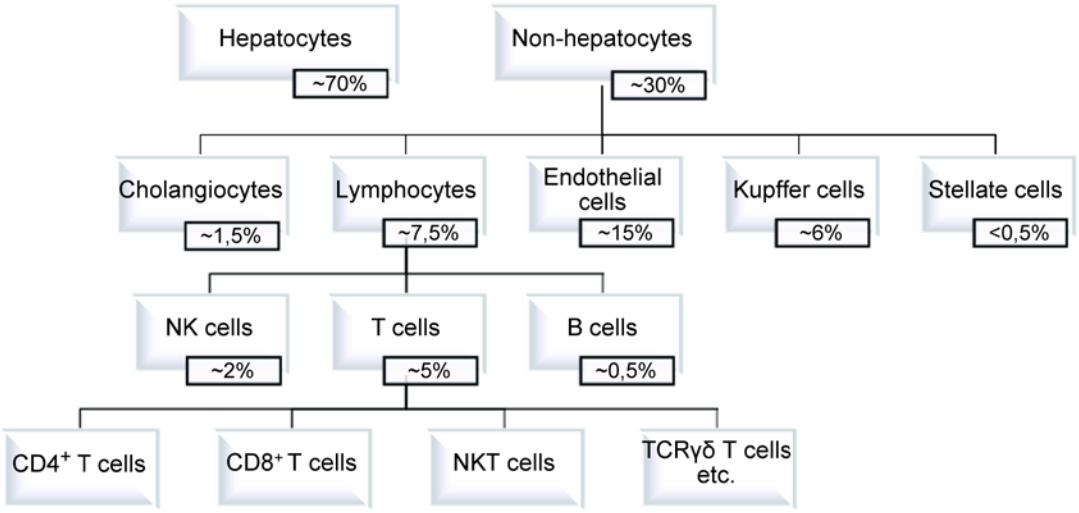

Figure 2. The proportion of parenchymal (hepatocytes) and non-parenchymal cells in a healthy liver. Adapted from [41].

1.1.3.1 Hepatocytes

The hepatocytes are rich in cellular organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus, a sign of active protein synthesis and secretion from these cells [43]. Hepatocytes have a large nucleus and about 25% of the cells are bi-nucleated which often results in polyploidy, suggested to be an important mechanism to restrict liver growth and prolong cell survival [44]. The hepatocellular membranes have a complex structure with different membrane sections with different biochemical composition and functional properties: the basolateral section (facing the sinusoids), the lateral (inter-cellular) section, and the apical section facing the bile canaliculi [36].

The hepatocytes possess a variety of different functions. They produce bile that is vital for the digestion of lipids. Many serum proteins i.e. albumin and blood clotting factors are synthesized by the hepatocytes and they also regulate the glucose homeostasis in the blood in response to glucagon and insulin signaling. The hepatocytes are also essential for the biotransformation of many endogenous substances, like different serum proteins, lipids and steroids. They also metabolize many exogenous substances, such as alcohol, chemicals and pharmaceuticals. Hepatocytes also play an important role in the hepatic immune response via the production of complement factors and acute phase proteins as a response to cytokine stimuli, like IL-6 (interleukin-6), IL-1β and TNFα, produced by Kupffer cells and endothelial cells [36]. Hepatocytes have also been reported to acquire antigen presenting skills [45] and are generally considered to be both the target and inducer of the innate immune response [41].

The liver has a remarkable regenerating capacity both via proliferation of hepatocytes [46] and via activation and differentiation of oval cells [47, 48]. Growth factors and cytokines, such as HGF (hepatocyte growth factor), TGF-β, FGF1, IL-6 and TNFα, released by Stellate cells and Kupffer cells respectively, has shown to have hepatoprotective effects and to stimulate liver regeneration [40, 42, 49-51].

Additionally, TGFβ signaling, which under normal conditions keeps the hepatocytes in a quiescent state, is suppressed during injury [47].

1.1.3.2 Oval cells

Oval cells are adult hepatic progenitor cells, expressing markers of both hepatocytes and biliary cells. They populate the canals of Hering, the zone between the periportal hepatocytes and the biliary cells of the smallest intrahepatic bile ducts [52]. In a healthy human liver the oval cells are quiescent and present only in small numbers. Contrary, during severe and prolonged liver damage when hepatocyte proliferation is compromised, the oval cells are activated, start proliferating and infiltrate into the parenchyma, giving rise to both hepatocytes and biliary cells [42]. It has been shown that various cytokines, such as TNFα, released from Kupffer cells [53] may have a role in oval cell activation [42, 54]. Stellate cells have also been suggested to be involved in the proliferation and differentiation of oval cells by secretion of several potential hepatocyte mitogens, such as HGF [42, 49]. Oval cell maintenance and liver regeneration activities are also regulated by extracellular matrix components [55, 56]. In rats, cytokines such as OSM are shown to inhibit oval cell proliferation, inducing differentiation [57].

1.1.3.3 Cholangiocytes

Cholangiocytes are epithelial cells lining the hepatic bile ducts and via different secretory and absorptive processes they modify the composition, pH and fluidity of the bile [36]. They also have an active immunologic role in both the innate and adaptive immune responses by interacting with immune cells through expression of adhesion molecules and antigens. When activated by pro-inflammatory cytokines, like TNF-α and IL-6, they secrete chemoattractant cytokines, such as IL-8 and MCP-1 (monocyte chemoattractant protein 1) [58]. Many drugs that induce a hepatic toxic response or chronic inflammation, result in dysfunction of the bile formation and bile flow, ultimately leading to cholestasis [59].

1.1.3.4 Sinusoidal endothelial cells

The hepatic sinusoids are lined by fenestrated endothelial cells. The basolateral surface of the hepatocyte is separated from endothelial cells by the space of Disse. The fenestration allows efficient transfer of proteins and other macromolecules between the blood and the hepatocytes. The fenestration also facilitates the communication between cells in the sinusoidal lumen and the hepatocytes as well as other cells in the space of Disse [41, 60]. The sinusoidal endothelial cells play an important role in the hepatic immune response as they participate in the clearance of antigens from the circulation by receptor mediated endocytosis, cytokine secretion and by antigen presenting capacities [60]. They also collect and present antigens originating from hepatocytes [41]. The regulation of endothelial antigen presentation and their role in induction of apoptosis of activated T cells play an important role for the immunologic tolerance in the liver [61].

1.1.3.5 Stellate cells

The hepatic stellate cells, or fat storing cells, are spindle-shaped cells located in the space of Disse, with extensions into the inter-hepatocellular space. They have an important role in storage and transportation of retinoids (vitamin A compounds) [62] and the have the ability to secret different components of the extracellular matrix (ECM), like collagen, proteoglycans and laminin, all essential for many hepatocellular functions [63]. Stellate cells also play a role in hepatic immunoregulation as they are known to express Toll-like receptors for LPS stimuli [64]. Activated stellate cells can amplify an inflammatory response in the liver by secretion of cytokines and chemokines [49] as well as by antigen presentation [65-67]. When activated, the stellate cells become depleted of vitamin A and via fibrogenic activities they start synthesizing large amount of ECM components, including collagen and adhesive glycoproteins [49, 68]. Chronic liver injury may lead to overproduction of ECM by the stellate cells which ultimately results in liver cirrhosis [47].

1.1.3.6 Kupffer cells

Kupffer cells together with lymphocytes constitute the major part of the hepatic immune cells. Kupffer cells are resident liver macrophages with migratory, phagocytic, inflammatory and antigen presenting capabilities, believed to be derived from circulating monocytes [41, 69]. The major part of the Kupffer cells are found around the periportal veins where the cells are larger and more active in phagocytosis compared to those found around the central veins [70]. Kupffer cells reside in the sinusoids where they are in close contact with passing lymphocytes as well as with the hepatocytes via the space of Disse [41]. They constitute the first line of defense and their location provides effective clearance of endotoxins like LPS and other infectious agents and [71]. Thus, Kupffer cells have important regulatory function in the pathophysiological state of the liver. When activated they release a cascade of various pro- and anti-inflammatory mediators such as interferons, interleukins (i.e. IL-1β, IL-4, IL-6, IL-10), nitric oxide and reactive oxygen species (ROS) [40, 50, 72]. Moreover, Kupffer cells are the main hepatic producers of TNFα, an important mediator of liver injury [40]. Activated Kupffer cells also stimulate other immune cells, like natural killer (NK) cells and natural killer T (NKT) cells, as well as recruit neutrophils by secretion of the chemotactic cytokines, like IL-8 [73]. Kupffer cells may also stimulate hepatocytes to produce IL-8, further increasing the chemotactic response [74].

1.1.3.7 Lymphocytes

Liver resident lymphocytes are regarded liver specific and differ phenotypically from lymphocytes found in the general circulation [69]. They reside predominantly in the periportal regions and the composition of the lymphocytes populations vary both with age and gender [75, 76]. Lymphocytes play a key role in the adaptive immune response and usually require antigenic stimulation. Like Kupffer cells they can produce both pro- (mainly IFN-γ but also TNFα and IL-2) and anti-inflammatory cytokines (i.e. IL-4 andIL-10) in response to agents, such as LPS [40, 69].

Lymphocytes consist of three major cell types; NK cells, T cells, and B cells (Figure 2). The NK cells have spontaneous cytotoxic activities against tumors, bacterial-, parasite-

and virus-infected cells and are critical to the innate immune system [77, 78]. The NKT cells are the predominant T cell type and express the NK cell marker as well as other T cell receptors (i.e. the αβT or the γδT cell receptor), recognizing antigens in association with the HLA (human leukocyte antigens) class I molecules on antigen presenting cells [41]. NKT cells are also able to distinguish a more limited variety of antigens which is not HLA dependent, such as bacterial and viral non-peptide antigens [79]. NKT require the presence of cytokines such as IL-2 and IL-12 for activation of their cytotoxic [77, 78]. NKT cells have been shown to scan the liver sinusoids by crawling within the sinusoids and stopping upon T cell antigen receptor activation [80]. The $CD8^+$ cytotoxic T cells and the $CD4^+$ helper T cells both display the αβ T cell receptor, recognizing antigens presented in association with HLA class I and class II molecules respectively. $CD8^+$ cytotoxic T cells target virally infected cells and tumor cells, destroying them via release of various cytotoxins. Activated $CD4^+$ helper T cells proliferate, differentiate and regulate different type of immune response such as maturation of B cells and activation of $CD8^+$ T cells and Kupffer cells by secretion of various cytokines like IL-4, IL-5, IL-2, TNF-α and IFN-γ [81]. Importantly, T (and B) cells possess a "memory", which makes them respond more vigorously when re-exposed to the same antigen [82]. During non-inflammatory conditions, stimulation of sinusoidal endothelial cells and Kupffer cells do not induce a T cell response but rather induce the secretion anti-inflammatory cytokines, like IL-10, contributing to immunological tolerance [83, 84]. Activated B cells are antibody-secreting cells that reside only in small numbers in a healthy liver [41].

1.1.4 Biotransformation in the liver

The liver plays a crucial role in the metabolism of several endogenous substances but also of exogenous xenobiotics, such as medical drugs. Many drugs are lipophilic and require metabolism to increase their water solubility, facilitating excretion via bile and urine. Some drugs also require metabolism in order to generate the active pharmacological compound [36]. Hepatic biotransformation is generally divided in two processes: phase I and phase II reactions. Additionally, hepatic transporters are generally considered to constitute phase III processes and play crucial roles in drug absorption, distribution and excretion (Figure 3) [85]. Thus, metabolism is a multi-step process that involves multiple reactions [86]. Phase I reactions (mainly redox reactions) generally increases the polarity of the substrate and as a consequence these reactions often generate reactive intermediates that could be toxic to the cells [87, 88]. The oxidative phase I processes, mainly catalyzed by the cytochrome P450 enzyme family, are by far the most important reactions in drug metabolism and will be discussed in more detail later. Some phase I products are excreted but most undergo a subsequent phase II reaction where an endogenous substrate, such as glucoronic acid or glutathione, is added forming a polar conjugate. This generally renders a more water soluble and less reactive derivate that readily can be excreted [86, 89]. Thus, the status of the drug metabolizing enzymes influences the metabolite formation and, hence, the toxic capacity of the drug. Moreover, regulation of several phase I and phase II enzymes has been shown to alter the expression of many phase III transporters thereby affecting the excretion of xenobiotics [85].

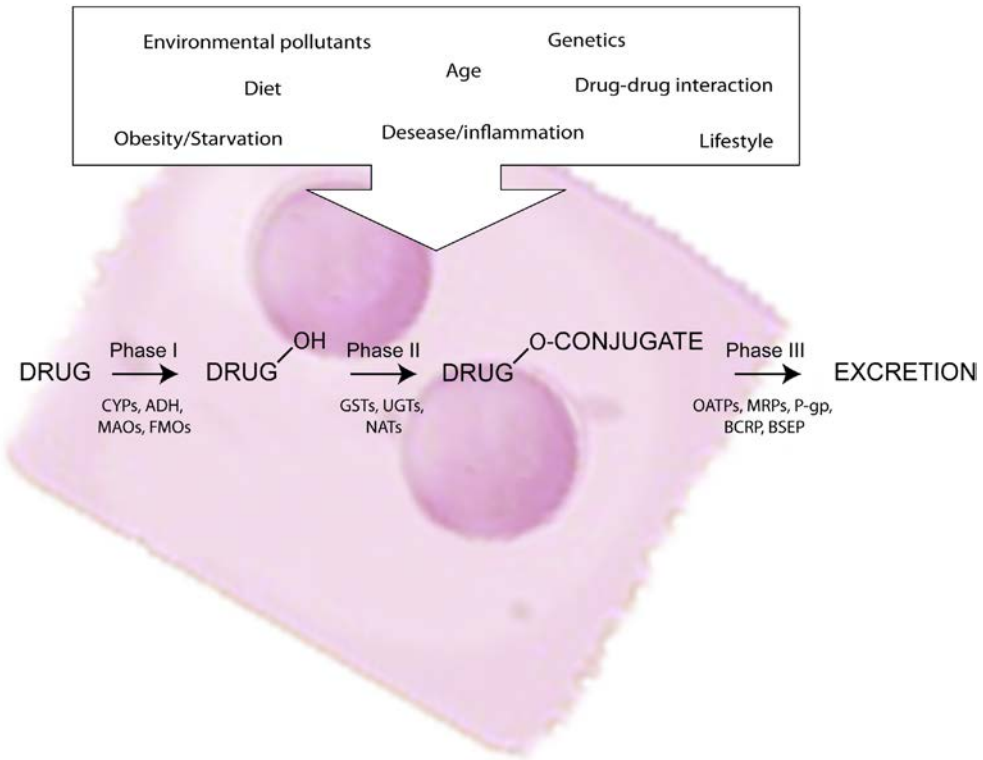

Figure 3. Hepatic biotransformation is generally divided in two processes; phase I and phase II reactions. Phase I reactions largely increase the polarity of the substrate, making the compound more reactive. Subsequent, phase II reaction often forms polar conjugates, which are less reactive and can be readily excreted. Additionally, transporters are generally considered as a phase III process since they play crucial roles in drug absorption, distribution and excretion. Many different factors may affect the expression of the enzymes and transporters involved in biotransformation of drugs. CYP, Cytochrome P450; ADH, Alcohol dehydrogenase; MAO, Monoamine oxidase; FMO, Flavin-containing monooxygenase; GST, Glutathione S-transferase; NAT, N-acetyltrasferase; UGT, UDP glucuronosyltransferase; OATP, Organic anion transporting polypeptide; MRP, Multidrug resistance-associated protein; P-gp, P-glycoprotein; BCRP, Breast cancer resistance protein; BSEP, Bile salt export pump.

1.1.4.1 Cytochrome P450s

The cytochrome P450 superfamily are heme-containing enzymes [90-92] involved in the metabolism of a wide variety of endogenous and exogenous compounds, such as steroid hormones, fatty acids and medical drugs [93]. The CYP enzymes, localized in the endoplasmic reticulum and in the mitochondria, are mainly expressed in the liver but significant amounts are also present in the intestine, as well as in kidney and lung [94]. The CYP catalyzed reactions are dependent on molecular oxygen where one oxygen atom is reduced to water while the second oxygen atom oxidizes the substrate, rendering a more polar product. This process requires electron transfer from reduced $NADP^+$, which is mediated mainly by cytochrome P450 reductase (POR), although in several cases cytochrome b_5 has also shown to be involved [95, 96].

The CYP enzymes are divided into different families, subfamilies and individual enzymes based on similarities in their amino acid sequences [97, 98]. The CYP enzymes belonging to family 1, 2 and 3 are the most important ones regarding

metabolism of drugs and other xenobiotic substances [87, 99] and are estimated to account for about 70-75% of all phase I metabolism of drugs used in the clinics [100, 101]. Many of the most clinically relevant CYPs are highly polymorphic [102] which can result in altered enzyme activity and often translates into inter-individual differences regarding therapeutic effect and susceptibility to drug induced toxicity [100, 103]. More information regarding genetic polymorphisms of the different CYP families and their phenotype can be found in "the human CYP allele nomenclature database" (http://www.cypalleles.ki.se). Apart from genetic variability, other factors such as age [104], gender [105], general health status [106] and concomitant usage of several drugs (both pharmaceutical and alternative) [100] may also affect CYP enzyme expression. Moreover, our metabolic phenotype is also affected by different environmental factors, such as our diet and lifestyle choices, i.e. cigarette smoking and alcohol consumption [106-108] (Figure 3).

1.1.4.1.1 CYP3A4

The human *CYP3A* genes, *CYP3A4*, *CYP3A5* and *CYP3A7* are the most abundant CYPs in human liver, accounting for about 30-40% of the total CYPs present [101, 109] Evans 1999). CYP3A4 accounts for the larger part of the CYP3A enzymes [102, 109] and is clinically the most important one. *CYP3A7* is predominantly expressed in fetal liver [110]. CYP3A5 has similar substrate specificity as CYP3A4 but is only detectable in 20-30% of the human population where it generally is expressed in lower levels [110, 111]. CYP3A4 is involved in the metabolism of a wide range of endogenous substrates, such as steroid hormones [112, 113] and bile acids [114], but also many exogenous compounds [98]. Importantly, CYP3A4 is involved in the metabolism of about 50% of all marketed drugs, such as midazolam, verapamil, and simvastatin [111].

CYP3A4 is a polymorphic enzyme [109, 115] and the large inter-individual variation has been suggested to be caused by alterations in the promoter region [116-118]. To date, more than forty allelic variants have been identified and some are reported to affect the function of the enzyme (http://www.cypalleles.ki.se), however, none are present at high enough frequency as to explain the inter-individual variation in catalytic activity seen in the human population [102]. It is more likely that the metabolic variability could relate to non-genetic factors such as health status, gender, diet and environmental factors [105, 106, 108]. CYP3A4 is also particularly susceptible to enzyme inducers, such as the antibiotic rifampicin and the herbal antidepressant St. John's wort, as well as inhibitors, like grapefruit juice and the antifungal drug ketoconazole [98, 119, 120]. Moreover, the level of induction can vary between individuals depending on the substrate [120]. Thus, drug toxicity due to altered CYP3A4 metabolic activity is relatively common in humans.

1.1.4.1.2 *CYP3A4* gene regulation

The first 13 kb of the *CYP3A4* 5'-flanking region have been thoroughly analyzed regarding its regulation [121]. In this region three distinct elements have been identified to, in a cooperative way, be important for the regulation of the gene; the proximal

promoter region (PROX), the xenobiotic-responsive enhancer module (XREM) [122, 123], and the far distal constitutive liver enhancer module (CLEM4) [124] (Figure 4).

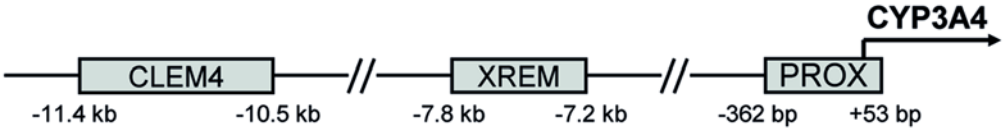

Figure 4. Schematic figure of the 5'-CYP3A4 promoter containing three important regulatory regions; PROX, XREM and CLEM4. Adapted from paper V.

The *CYP3A4* gene is transcriptionally regulated by the cross-talk between various transcription factors [106, 125], such as the nuclear receptors PXR (pregnane X receptor, NR1I2) [126-128], CAR (constitutive androstane receptor, NR1I3) [129], VDR (vitamin D receptor, NR1I1) [130] and GR (glucocorticoid receptor, NR3C1)[131]. These nuclear receptors interact with multiple regulatory DNA sites within the promoter region of the *CYP3A4* gene. Additionally, other liver-enriched transcription factors, such as hepatocyte nuclear factors (HNF) and CCAAT/enhance-binding proteins (CEBP), are also important for CYP3A4 regulation and essential for the constitutive expression [106, 124, 132, 133]. HNF4α, in particular, is known to be an important player in the regulation of *CYP3A4* by the interaction with PXR [124, 132] but also for the transcriptional regulation of *PXR* [33].

Recently a unique study was published showing highly variable CpG methylation frequencies in several important CYP3A4 transcription factor binding sites [134]. Two single CpG sites were identified as significantly associated with CYP3A4 expression [134], suggesting that epigenetic variations may be of importance for the inter-individual differences in gene expression [102]. Moreover, Kacevska *et al.* showed that the methylation pattern for the CYP3A genes differed between adult and fetal livers [134], indicating that epigenetic modifications regulate the developmental switch. This is supported by a study done in mice where epigenetic histone modifications exhibited dynamic changes during liver development [135]. Several studies also report of post-transcriptional regulation of CYP3A4, both via direct targeting [136] but also via indirect targeting of CYP3A4-regulating transcription factors, such as PXR [137-139] and VDR [136]. Additionally, CYP3A4 may also be subjected to ubiquitination and degradation via PKA/PKC mediated phosphorylation [140].

1.1.4.1.2.1 PXR

PXR has one of the broadest ligand spectrums of the nuclear receptor superfamily with many endogenous substrates, such as steroids and certain bile acids, as well as exogenous compounds, like the antibiotic rifampicin. PXR is an important modulator of several key biochemical pathways, such as gluconeogenesis and beta-oxidation, and plays an important role in bile homeostasis by down-regulation of CYP7A1 [141, 142].

PXR is activated by many xenobiotic substrates and is vital for the metabolism/detoxification process [143]. PXR is a major transcriptional regulator of

CYP3A4 [126-128] but also of other important phase I genes, such as *CYP2C* [144] and *CYP2B* [145], phase II genes, like *UGT1A1* [146], and membrane transporters, like *MDR1* [147] and *MRP2* [148]. Importantly, PXR is estimated to be the nuclear receptor responsible for about 60% of all undesirable, clinically relevant drug-drug interactions involving CYP3A4, consequently playing a major role in the development of ADRs [128, 141]. PXR has also been suggested to play an important role in the molecular mechanism that links xenobiotic metabolism and inflammation. It is well known that the enzyme activities of several CYPs, including CYP3A4, are negatively affected by inflammatory mediators, like IL-6 and TNFα [106], negative regulating transcription factors, such as PXR, possibly via the inflammatory mediator NF-κB (nuclear transcription factor kappa B) [106, 149].

It is generally considered that PXR induction is ligand dependent and that activated PXR is translocated from the cytoplasm to the nucleus where it interacts with the promoter region of its target gene as a heterodimer with RXR (retinoid X receptor). However, there are still different opinions regarding the subcellular localization of unliganded PXR [150-152]. PXR regulates *CYP3A4* by binding to responsive elements composed of various repeats of the consensus motif AG(G/T)TCA. These repeats include direct repeats (DR) and everted repeats (ER) separated by different numbers of nucleotides [125]. PXR is known to bind strongly to an everted repeat separated by six base pairs (ER6) located in the PROX region [128], in the XREM region [122] and in the far distal CLEM4 region [153], as well as to direct repeats separated by 3 (DR3) or 4 (DR4) base pairs the distal XREM region [122, 154].

PXR regulation has been studied extensively but is far from being fully elucidated. The transcriptional activity of PXR is known to be regulated by different co-repressors, such as SMRT (silencing mediator of retinoid and thyroid receptors), COUP-TF (chicken ovalbumin upstream promoter transcription factor) and NcoR (nuclear receptor co-repressor) [155-157], as well as various co-activators, such as HNF4α [132], PGC-1 (Peroxisome proliferator-activated receptor gamma co-activator) and SRC-1 (steroid receptor co-activator) [158]. The differential recruitment of co-factors has shown to be ligand-dependent [159]. In addition, PXR has shown to be post-transcriptional regulated by miRNA (miR-148a) [137] as well as by post-translational mechanisms, like ubiquitination [156], acetylation [155], phosphorylation by various kinases [138, 139, 160] and epigenetic regulation by protein arginine methyltransferase1 (PRMT1) [161]. Adding to the intricate regulation of PXR, several SNPs has been described for the *PXR* gene [123, 162, 163] along with various identified splice variants [107, 164, 165] which are suggested to play a part in the inter-individual variations in basal and inducible expression of CYP3A [150, 163, 165].

1.2 HUMAN LIVER CELLS IN RESEARCH

1.2.1 Primary hepatocytes

Primary human hepatocytes generally express all the drug metabolizing enzymes and transporters found in human liver which makes these cells the closest model to the *in*

vivo liver. This is why primary hepatocytes are considered the golden standard in drug development and toxicological studies [166]. However, primary hepatocytes are phenotypically instable and when they are isolated from their *in vivo* microenvironment and put in 2D cultures they rapidly de-differentiate into in a population of adult liver progenitors [167], loosing many of their liver specific functions, in particular CYP enzyme levels [168]. In addition, 2D culture limits the survival of the cells to only 1-2 weeks [169]. However, by culturing primary hepatocytes in a sandwich culture of collagen or matrigel, the hepatocyte life span, morphology and specific hepatic functions can be preserved for longer period of time [169, 170]. Unfortunately, the sometimes low availability of fresh human liver samples compromise the use of primary hepatocytes in routine testing. Moreover, the resected livers most often originate from medicated patients that may severely affect cell viability and specific functions. Regarding donated livers, the patients have often been subjected to various pharmaceuticals, e.g. for the treatment of brain injury, again potentially affecting the expression of various drug metabolizing enzymes [89]. Cryopreserved hepatocytes are often used as they are available and phenotypically characterized which facilitates their use in routine research [171]. Moreover, pooled cells from several donors are available which reduce inter-donor variability. However, these cells are expensive and share the same limitations as freshly isolated hepatocytes regarding loss of liver specific functions in culture.

1.2.2 Hepatoma cell lines

Due to the scarce number and limited proliferation potential of human hepatocytes along with high variability of drug metabolizing enzyme expression in different preparations, hepatocellular carcinoma-derived cell lines, such as HepG2 and Huh7, are frequently used to get insight in mechanistic pathways regarding metabolism and toxicity. Hepatoma cell lines are easy accessible, easy to culture and provide a cell source of high yield. Moreover, they have a more stable phenotype which makes them more useable in routine testing [89]. However, these cell lines generally contain very low levels of drug metabolizing enzymes, such as CYPs [172], and often require modifications, like transfections and/or enzyme induction [168, 173]. Lately a new hepatoma cell line, HepaRG, was generated. After DMSO treatment these cells has been shown to differentiate into a mixed cell population of both hepatocyte- and biliary-like cells [174] with pronounced expression of some CYPs, UGTs and transporter activities [175, 176].

1.2.3 Stem cells

Stem cells are undifferentiated (unspecialized) cells with the ability to develop into many different types of cells and they possess unlimited replication capacity [177]. Generally there are two different types of stem cells, embryonic and somatic (adult) stem cells, with different characteristics and potentials [177]. Recently, researchers have been able to successfully reprogram fully differentiated somatic cells into cells with stem-cell like properties, called induced pluripotent stem cells (iPSC) [178].

1.2.3.1 Embryonic stem cells

The breakthrough for stem cell research came about 30 years ago with the first successful isolation of an embryonic stem cell line from a mouse embryo [179, 180]. In 1998, the first generation of an *in vitro*, multi-passaged, culture of human embryonic stem cells (hESC) was reported [181] and since then many different protocols have been developed for the establishment, propagation and characterization of hESC. Embryonic stem cells represent the inner cell mass of the blastocyst in the earliest stage of the embryo development and are characterized by the expression of various transcriptional factors such as OCT-4 (octamer-binding transcription factor 4) [182], SOX2 [183] and NANOG [184] (Figure 1). These cells are pluripotent and can generate all three germ layers, thus capable of differentiating into any kind of cell in the human body [181, 185]. With these unique properties, these cells provide a highly interesting model system for basic research on embryonic and organ development, as well as a hepatic cell source for drug discovery and toxicology studies [186, 187]. In the future, these cells might also be used in clinical therapies [186, 188]. During the recent years, much effort has been put into the development of effective protocols for hepatic differentiation of hESCs, largely based on what is known about the embryogenesis (Figure 1) [167, 187, 189-193].

1.2.3.2 Hepatic somatic stem cells

Somatic stem cells have a more limited differentiation potential compared to hESC but has an important role in tissue homeostasis and injury repair in the multicellular organism [177]. The presence of hepatic stem cells (oval cells) were first discovered in fetal mice livers [194] and was later also isolated from human adult livers [195]. Oval cells are multipotent stem cells that can give rise to both hepatocytes and biliary cells [42]. Hepatic oval cell lines have been generated that retain the progenitor cell features expressing markers for both cholangiocytes and hepatic progenitors after long term cultivation and serial passages [196]. These cells might thus serve as an expandable hepatic cell source for research and for cell-based therapy [197, 198].

1.2.3.3 Induced pluripotent stem cells

In 2006, a Japanese research group successfully reprogramed adult mouse fibroblasts into induced pluripotent stem cells (iPSC) by introducing four transcription factors: c-Myc, Oct3/4, Sox2 and Klf4, by retroviral transduction [178]. A similar approach was also successfully performed with human fibroblasts (Takahashi 2007) and later using other cell types from both human and mouse [199]. The iPSC are stem cell-like regarding morphology and characteristics, such as pluripotency and genetics, expressing a number of stem cell biomarkers [199]. Several groups have subsequently been able to successfully generate hepatocyte-like cells from iPSC [200-202]. The iPSC technology is promising with a future potential in patient- and disease-specific therapy [199]. However, in order for these cells to be used in a clinical application several important issues has to be addressed, such as somatic origin memory, donor-dependent variations, low reprogramming efficiency, risk of potential teratoma formation, safety concerns regarding transduction delivery methods and the presence of transgenes, like oncogenes [199, 203].

1.3 DRUG INDUCED LIVER INJURY

DILI represents a major challenge for the public health care as well as for the pharmaceutical industry. Liver injury is a serious ADR, estimated to account for about half of all cases of acute liver failures, a lethal condition that often requires liver transplantation [204]. Even though paracetamol poisoning (intentional and unintentional) accounts for the major part of DILI [13], as much as 16% of all acute liver failures are of idiosyncratic nature making DILI one of the most commonly reported ADR [205, 206]. Contrary to intrinsic (dose-dependent) drug reactions, idiosyncratic drug reactions only occur in a small subgroup of patients, are usually independent of dose and often have long latency periods [13]. Due to safety regulations, a new compound has to be validated in both in animals (*in vivo*) and in cell models (*in vitro*) before entering clinical testing. However, idiosyncratic reactions are usually not predicted by the pre-clinical models or even during pre-marketing clinical trials [207]. During the last decade's, toxic effects on the liver has been one of the most cited reasons for regulatory actions concerning drugs, including boxed warnings and restricted marketing [9, 208]. Additionally, in a report from 2003 it was estimated that as much as 50% of all approved drugs withdrawn from the market were related to toxic effects on the liver [206].

1.3.1 Immune mediated hepatotoxicity

There are many drugs on the market today, i.e. diclofenac and flucloxacillin, that are known to have associations with DILI in certain patients and it has become increasingly evident that inflammatory event plays a significant role, involving both the innate and adaptive immune system [13]. Many different factors may trigger an immunological response and influence the toxicological outcome during drug treatment: the levels of reactive metabolites formed causing stress and/or cell damage [100], adducts generated by binding of the drug or metabolite to cellular proteins and macromolecules or an underlying infection [209].

The Kupffer cells are known to be especially important in the progress of DILI [13]. Kupffer cells respond to bacterial endotoxins, like LPS, via the TLR4 (toll-like receptor) expressed on most liver cells [41]. The activation results in the production of a range of inflammatory mediators, such as cytokines and ROS. Some of these mediators, like IL-10 and IL-6, may work in a hepatoprotective manner whereas some, such as and TNFα and IL-1β, often contribute to the progression of liver injury where other cells are activated, adding on to the immunological response [82]. TNFα released from activated Kupffer cells may also induce apoptosis and necrosis in hepatocytes via production of nitric oxide and ROS as a result of mitochondrial dysfunction [209]. During liver insult, targeted cells, such as hepatocytes and sinusoidal endothelial cells, have the ability to present antigens to lymphocytes which adds to the immune response by the activation of the cytotoxic cells but also of memory cells which may potentiate the response whenever re-challenged with the drug [41, 210].

During the recent years, a number of idiosyncratic DILI-related drugs have shown striking association with specific HLA alleles (Table 1). HLAs are cell surface glycoproteins with the role to present peptide antigens to T cells, thus playing an essential role in the innate and adaptive immune system [36]. The HLA class I proteins (i.e. A, B, and C) are expressed on most cell types and presents antigens that mainly activates $CD8^+$ cytotoxic T cells. HLA class II proteins (i.e. DR, DQ, and DP) are generally expressed on antigen presenting cells and often activate $CD4^+$ T helper cells. The HLA molecules are highly polymorphic with allele frequencies that vary between different populations and ethnic groups which causes inter-individual variability in susceptible to certain pathogens [211]. For some drugs FDA (US Food and Drug Administration) has even suggested screening of patients for specific HLA alleles before use, as done for carbamazepine (HLA-B*15:02) and abacavir (HLA-B*57:01) to avoid hypersensitivity reactions [212].

HLA Allele	Compound	Therapy area	Odds ratio	Reference
B*57:01	Flucloxacillin	Antibiotic	80.6	[213]
DRB1*07:01-DQA1*02:01	Ximelagatran	Anticoagulant	4.4	[214]
DRB1*15:01-DQB1*06:02	Co-amoxiclav	Antibiotic	2.8	[215]
DRB1*15:01-DQB1*06:02-DRB5*01:01-DQA1*01:02	Lumiracoxib	COX-2 inhibitor	5.0	[216]

Table 1. DILI- related drugs associated with specific HLA alleles in Caucasians.

1.3.2 *In vitro* methods to study DILI

Mono-cultures of primary hepatocytes are the most frequent used liver-specific *in vitro* model for drug metabolism and toxic evaluation [166]. These cell systems are valuable models as they possess the essential enzymes and transporters for the biotransformation pathways [217]. However, due to scarce availability of human liver tissue, hepatocytes of animal origin and cell lines are also frequently used with evident advantages with respect to their availability. Additionally, immortalized human hepatocytes with stable overexpression of various CYPs have also been described [204]. While these cell models are valuable for drug screening and toxicity, they do not always extrapolate to human biology. Due to limitations such as low metabolic capacity, species-specific mechanisms and inadequate extra-cellular milieu, the *in vitro* cell systems used today have rather low prediction of DILI.

Primary hepatocytes are highly dependent on tight cell-cell contact and organized cellular architecture, not only for the maintenance of their differentiated functions and organized tissue architecture, but also for the regulation of their proliferation status [218, 219]. So far, precision-cut tissue slices best meet this requirement where the organ structures are maintained together with expression of phase I and II enzymes and

required co-factors [220]. This culture technique has been further improved by continuous medium exchange [221], however, due to special technical requirements and skills together with the low availability of freshly human liver tissue, this method has limited applications. During the last decade several promising 3D culture systems have been developed, designed to better mimic the physiological conditions in the liver, with the aim to retain the hepatic functions of primary hepatocytes. Some examples are the use of various scaffolds [222, 223], bioreactor cultured spheroids [224, 225] and hollow fiber bioreactors [226-228] which all show improved hepatocyte function and maintenance, although with varying results. Many of these culture systems are perfused providing the cells with a continuous supply of nutrients and oxygen, important factors since the hepatocytes are highly susceptible to oxygen and nutrient limitations [226].

DILI is caused by multiple complex mechanisms and apart from the metabolic aspects, toxic onset after drug treatment often involves the interplay between several different types of cells [13]. Co-cultures between hepatocytes and epithelial cells [229] or hepatocytes and fibroblasts [230] have shown to improve the expression of biotransformation enzymes of hepatocytes in culture. Moreover, co-culture of primary hepatocytes and hepatic stellate cells [231, 232], hepatocytes and macrophages [233] and macrophages and cholangiocytes [234] have also been developed. All have been able to show toxic mechanistic interactions which might not have been achieved using conventional hepatocyte mono-cultures. This supports the fact that in order to study the relevant *in vivo* mechanisms, more advanced *in vitro* cell systems has to be developed, where cellular interaction, architecture and integrity is better preserved.

2 AIM

The aim of this thesis was to refine and further develop more sensitive, human, *in vitro* models and methods for better prediction of drug induced liver injury and the underlying mechanisms. The work presented here constitutes of two different parts:

1) To generate new sources of functional human hepatic cells.

- Cell-cell contact-promoted differentiation of the human hepatoma cell line Huh7 has been investigated.

- A stepwise, directed, differentiation protocol has been evaluated for hepatic differentiation of human embryonic stem cells.

2) To develop and evaluate new *in vitro* cell culture models to improve and maintain the hepatic functionality of the cells, better extrapolating to human liver biology.

- A human co-culture model, incorporating hepatocytes and monocytes, has been developed to evaluate the inflammatory aspects of drug induced liver injury.

- Metabolically active Huh7 cells have been generated to study the endogenous regulation of *CYP3A4*.

- A dynamic bioreactor system has been used for the evaluation of three-dimensional culture in the hepatic differentiation of human embryonic stem cells.

3 COMMENTS ON EXPERIMENTAL METHODS

3.1 CELLS

3.1.1 Human primary hepatocytes and liver tissue

Human liver tissue and primary hepatocytes used in in papers II, III, and IV were obtained from Sahlgrenska university hospital (Gothenburg, Sweden) and Karolinska university hospital (Huddinge, Sweden), originating from patients undergoing liver resection. All tissues were obtained by qualified medical staff, with donor consent and ethical approval. In paper II, purchased cryopreserved hepatocytes were used and plated on Collagen I coated cell culture dishes according to the manufacturer's instructions (In vitro Technologies).

3.1.2 Cell lines

In this thesis the monocytic cell line THP-1 (paper I) and the human hepatoma cell lines Huh7 (papers I, III and V) and HepG2 (paper III) were used and cultured according to manufacturer's instructions. The HepG2 cell line (ATCC) originates from 15 year old Caucasian American male [235] and the Huh7 cell line (HSRRB) from a 57 year old Japanese male [236], both with a well differentiated hepatocellular carcinoma. The THP-1 cell line (ATCC) was derived from a 1 year old male with acute monocytic leukemia [237].

3.1.3 Stem cell culture and differentiation

The human embryonic stem cell lines (hESC), used in papers II and IV (Cellectis Stem Cells, Cellartis AB), were derived from surplus human embryos from clinical *in vitro* fertilizations and characterized as previously described [238, 239]. The undifferentiated cells was cultured as a monolayer on mitotically inactivated mouse embryonic fibroblasts (MEFs) [238, 239] or under feeder-free conditions and enzymatically passaged regularly according to Cellectis defined culture protocols. By directed differentiation via definitive endoderm (DE), hepatic progenitors (PRO) and finally to hepatocyte-like cells (HEP), the developmental phases seen *in vivo* were mimicked. The induction of hESC into DE was initiated by a 24h pre-treatment in Cellectis proprietary pre-treatment medium, followed by a media containing various additives, such as Activin A and sodium butyrate. On day 7, the generated DE cells were passaged and cultured for 3 days in progenitor medium supplemented with FBS and growth factors BMP2 and 4, FGF1 and FGF2, followed by a serum-free media containing dimethyl sulfoxide (DMSO). On day 12 the PRO cells were passaged and further matured into hepatocyte-like cells by culturing in medium containing various supplements like Oncostatin M, HGF, dexamethasone, DMSO, insulin, and hEGF (human epidermal growth factor). For the 3D culture experiments the DE and PRO cells were inoculated in the bioreactor, on day 7 and 12 respectively. DE and PRO cells were also seeded in conventional matrigel-coated 2D cultures for parallel culture throughout the differentiation process.

3.2 *IN VITRO* CELL CULTURE MODELS

3.2.1 The co-culture model

In paper I we evaluated if the incorporation of monocytes in hepatocyte cultures could generate a more sensitive *in vitro* system for drug hepatotoxicity studies. Kupffer cells, derived from monocytes, are known to be involved in the development of drug-induced hepatotoxicity by the release of both pro- and anti-inflammatory mediators [240, 241]. We created a human co-culture system consisting of the hepatoma cell line Huh7 and the monocytic cell line THP-1 (Figure 5). Since there were no human Kupffer cell lines available at the time of the study, the THP-1 cell line was used. The Huh7 cells were seeded as an adherent monolayer in 12-well plates (Costar®) and after 24h the non-adherent THP-1 cells were seeded into a Transwell® insert with a 3µM porous polyester membrane (Sigma-Aldrich), physically separating the two cell types by 1 mm but allowing molecules to passively diffuse. The insert-model proved to be superior to other models we tested (cells in direct contact or separated by a layer of collagen) since it allowed the cells to be evaluated separately. Moreover, the THP-1 showed to be more responsive to troglitazone treatment in the insert model as evaluated by the expression of TNFα. The ratio between the Huh7 and THP-1 cells was titrated in attempt to take the *in vivo* ratio into account. A ratio of 2.5:1 (Huh7:THP-1) was used based on the amount of 100% confluent Huh7 cells and the lowest amount of THP-1 cells from which RNA could be extracted. The cells were cultured in a 1:1 mix of each medium.

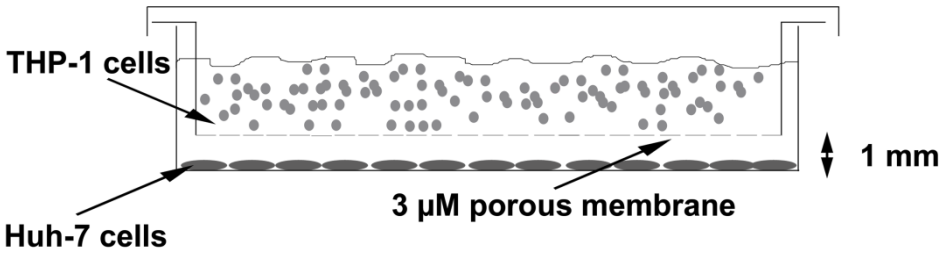

Figure 5. Schematic illustration of the co-culture model. The huh7 cells were seeded adherent in the bottom of the well. The THP-1 cells were seeded non-adherent in an insert with a 3µM porous membrane, separating the two cell-types by 1 mm. Figure from paper I.

A pair of thiazolidinediones was used as model drugs: Troglitazone (Rezulin®, Resulin® or Romozin®) and Rosiglitazone (Avandia®, Avandamet®, Avandaryl®). These drugs were developed for treatment of diabetes, sensitizing the action of insulin by acting as ligands for the nuclear peroxisome proliferator-activated receptor-γ (PPARγ) [242]. While rosiglitazone is not considered hepatotoxic, troglitazone has proved to cause idiosyncratic, hepatocellular injury in humans and was withdrawn from the market in 2000 [212].

3.2.2 The three-dimensional (3D) bioreactor technology

The multi-compartment, hollow fiber, bioreactor technology (Stem Cell Systems) [243] used in paper IV was originally developed for the clinic as a large-scale, bioartificial,

liver support system and has successfully been used to support the liver function of patients with acute liver failure [244]. The bioreactors consist of three independent bundles of hollow fiber membrane capillaries, interwoven into a 3D network which is enclosed by a polyurethane housing. The cells are inoculated in the cell compartment around the extra capillary space. Two of the capillary bundles are made of porous semipermeable polyethersulphone membranes for media perfusion and the third bundle consists of hydrophobic multilaminate membranes to enable gas exchange. In our lab, two lab scale bioreactors have been used with cell compartment volumes of 2 ml (Figure 6A) and 0.5 ml (Figure 6B), respectively. The bioreactor is connected to medical-grade, polyvinyl chloride tubing, creating a circuit that is integrated in a perfusion device (Figure 6C) where several bioreactor systems may be run in parallel. Peristaltic pumps generate a continuous flow of media through the bioreactor, removing waste products and providing the cells with nutrients and gas in a decentralized way and with high mass exchange rate which is of more physiological significance. Fresh medium is continuously added to the circuit and mixed with recirculating medium. A sample port allows sampling from the reticulating medium but also the addition of substrates to the circuit. The perfusion devise also maintain controlled culture conditions for the cells regarding temperature (37ºC), oxygenation and pH regulation (by CO_2) which may be adjusted manually.

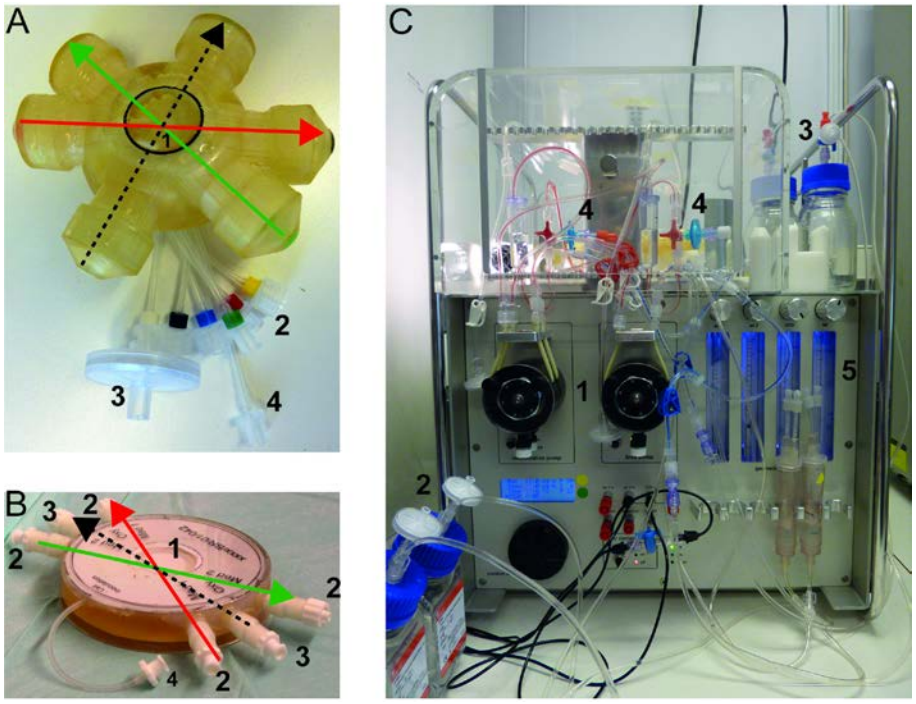

Figure 6. The three-dimensional, perfused, bioreactor technology [243]. The multi-compartment, perfused, hollow fiber bioreactor with a A) 2 ml or B) 0.5 ml cell compartment. 1) Cell compartment. Connections for 2) medium and 3) gas perfusion. 4) Port for cell inoculation. Filled arrows show direction of medium flow through the bioreactor, the red and the green each representing a separate bundle of capillaries. The dotted arrow indicates the gas flow through the bioreactor. C) The perfusion system with two separate bioreactors mounted. The bioreactors are connected to tubing for medium recirculation. 1) Speed-adjustable peristaltic pump units. 2) Bottles for addition of fresh medium and 3) collection of waste medium. 4) Sampling ports that enable sampling as well as injection of substrates. The temperature within the perfusion circuit is maintained at 37ºC. 5) The gas supply (air and 5% CO_2) may be manually regulated by a gas mixing unit.

3.3 GENE EXPRESSION ANALYSIS

3.3.1 Real-time PCR

Real-time polymerase chain reaction (RT-PCR) is a sensitive and quantitative method to determine the expression of certain genes in a sample. Gene specific primers containing fluorescent molecules are used and the progress of the PCR reaction can be monitored by the fluorescence intensity where the amount of fluorescence is directly proportional to the number of transcripts in the starting material. In this thesis two different real time methods have been used. In the SYBR Green method used in paper I, a non-specific, instable, fluorescent molecule were used that intercalates with the double-stranded DNA formed during the RT-PCR reaction. The binding of the molecule to the DNA changes the configuration of the SYBR Green and it starts emitting fluorescence. The TaqMan assay used in papers II, III, IV and V contains a sequence-specific oligonucleotide probe labeled with a fluorescent reporter. The probe is quenched when it is intact but during the PCR reaction when the gene-specific double stranded product is formed, the quencher is cleaved away and the probe starts emitting fluorescence. The TaqMan method is more sensitive since only the amplification of the target gene is detected whereas the SYBR Green molecule also binds to unspecific targets such as primer-dimers. The relative quantification of the different genes investigated was determined by adjusting the amount of transcript to housekeeping genes such as GAPDH and TBP. The relative levels in the samples compared with levels in control samples were defined by the comparative CT ($2^{-\Delta\Delta Ct}$) method as described by Livak and Schmittgen [245].

3.3.2 PCR Arrays

The PCR Arrays are medium-throughput RT-PCR assays that generate several different gene-specific products under uniform cycling conditions. The TaqMan Low Density Array (Applied Biosystems) used in paper III can simultaneously perform 12 to 384 TaqMan RT-PCR reactions pre-loaded in a microfluidic card format. These arrays can be custom made to include any TaqMan gene expression assay and each array can evaluate up to eight samples at the same time. The RT² Profiler PCR Array (Qiagen) used in paper I is a SYBR Green-optimized 96-well plate assay designed for the investigation of a panel of specific pathway-related genes. The relative quantitation of the individual target genes was generated using the comparative CT method as described above.

3.3.3 Affymetrix array

With the use of the microarray technology the expression of thousands of genes can be monitored simultaneously in a sample. Several different types of microarrays exist on the market. In papers IV and V we used the Affymetrix human GeneChip® ST (Sense target) 1.0 and 1.1 whole transcript arrays, respectively. These Affymetrix arrays are *in situ* synthesized, miniaturized, oligonucleotide probe arrays [246] with millions of immobilized, well-annotated, exon based probes which are designed to be distributed throughout the entire length of each transcript [247]. The principle behind the microarray technology is base pairing of two complementary sequences, the

immobilized probe on the array and the mobile target transcripts in the sample. The general procedure for a gene array experiment involves the conversion of the sample RNA to fluorophore labeled cDNA via reverse transcription. The labeled cDNA is then hybridized onto the array and scanned. The fluorescence intensity, directly proportional to the number of transcripts corresponding to each gene, is detected and the expression level of the gene is quantified. The obtained data was analyzed using DAVID (Database for Annotation, Visualization, and Integrated Discovery, www.david.niaid.nih.gov) in order to interpret the data in a more biological way [248, 249]. The microarray technology has had a remarkable impact on gene expression analysis since it was developed in the early 1990s. However, there are several issues to be aware of when using this technology that might bias the final result. Some examples are: relatively poor sensitivity in detecting low expressed transcripts, cross-hybridization, cross-platform inconsistency and variations in experimental procedure [250].

3.4 SUBCELLULAR FRACTINATION

3.4.1 Microsomes

Microsomes used in papers III and V consist of fragmented endoplasmic reticulum, the site where most of the drug metabolizing CYPs is located, which makes the microsomes a valuable tool for *in vitro* research regarding drug metabolism and drug-drug interactions. For preparation of microsomes the cells were lysed in buffer containing glycerol for maintaining the structure and activities of the proteins. The homogenate were centrifuged at 10,000 x g for removal of undisrupted cells, nuclei and mitochondria. The microsomes were then isolated from the supernatant by precipitation at 100,000 x g centrifugation [251].

3.4.2 Nuclear and cytoplasmic extracts

Nuclear and cytosolic extracts from cultured hepatoma cells were used in paper V. The cells were washed and re-suspended in hypotonic buffer to swell the cells and the cells were then lysed using the detergent NP-40. The nuclei were collected by centrifugation and the supernatant was saved as the cytosolic extract. After incubation of the nuclei in a high salt buffer and subsequent centrifugation the nuclear extract was collected.

3.5 CLONING AND TRANSIENT TRANSFECTION

In paper V, the CYP3A4 regulation was studied by the use of reporter constructs containing the three CYP3A4 5'-flanking regulatory regions; PROX (P), XREM (X) and CLEM4 (C). The different promoter constructs were created by PCR with a plasmid containing the -12.5 kb to +51 5'-promoter region of CYP3A4 [252] as a template. The regions were inserted in a pGL3 basic vector (Promega) using different restriction sites and the various constructs were verified by DNA sequencing. The full length promoter construct CYP3A4-PXC-luc containing all three regulatory regions is shown in figure 7.

For the plasmid transfection experiments, lipofection by Lipofectamine™ LTX with PLUS™ Reagent (Invitrogen) was used. During lipofection, nucleic acids are introduced into the cell with the use of a cationic lipid formulation that forms liposomes by binding with the nucleic acids. The liposome merges with the phospholipid bilayer of the cells, releasing the nucleic acid into the cell. The firefly luciferase enzyme was used as the construct reporter gene and co-transfected with a plasmid encoding the Renilla luciferase enzyme as a reference for normalization of the gene expression. For the gene silencing experiments in paper V, siRNA transfection was done using DharmaFECT1 (Thermo Scientific) containing lipids specially formulated to deliver siRNA. The cells were treated with target specific siRNA or a non-targeting siRNA pool as a negative control. Soon after being introduced into the cell, the ~20 bp long double-stranded siRNA molecules activate the RNA-induced silencing complex (RISC). Guided by the antisense strand of the siRNA, RISC degrades the targeted mRNA, preventing the synthesis of the protein, thus silencing the target gene.

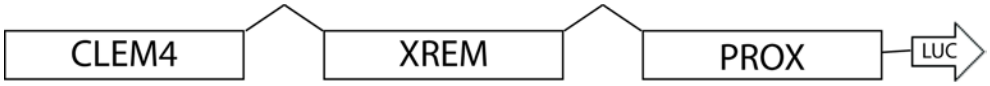

Figure 7. Schematic illustration of the CYP3A4-PXC-luc reporter construct containing three important regulatory regions in the 5'-CYP3A4 promoter; the PROX, XREM and CLEM4 site.

3.6 IMMUNOLOGICAL TECHNIQUES

In papers II, III, IV and V, proteins were visualized by different immunofluorescent techniques using antibodies that bind to the epitope of a specific protein. Secondary antibodies, conjugated to horseradish peroxidase or a fluorophore were then used to recognize the primary antibody using chemiluminescent or fluorescent techniques.

3.6.1 Immunoblotting

The denaturated proteins were separated on a SDS-polyacrylamide gel by electrophoresis (SDS-PAGE) depending on the length of the charged polypeptide [253, 254]. The proteins were then blotted to a nitrocellulose membrane and detected using antibodies that were visualized by chemiluminescence.

3.6.2 Immunohistochemistry

The samples from the bioreactor were treated with formaldehyde, cross-linking the proteins. Fixation with formaldehyde may alter the antigenicity of proteins and might require antigen retrieval, unblocking the cross-linked proteins. The tissue was dehydrated with increasing concentrations of ethanol followed by xylene which is miscible with the paraffin embedding media. After paraffin embedding the sample was sectioned. In order to allow water soluble dyes to penetrate the sections, the paraffin wax was removed and the sample rehydrated. Selected antibodies were then used to visualize certain protein by fluorescence. The sections were routinely stained hematoxylin and eosin for visualization of nuclei and cytoplasm, respectively.

3.6.3 Immunocytochemistry

Immunocytochemistry, a similar method as immunohistochemistry, was used to determine the presence and subcellular localization of specific proteins or antigens in the cultured cells. The cells were grown on cover slips, fixed in formaldehyde, and permeabilized using Triton X-100. The proteins were then detected using fluorescent conjugated antibodies and the nucleus was visualized with DAPI (4, 6-diamidino-2-phenylindole) staining.

4 RESULTS AND DISCUSSION

4.1 NOVEL HUMAN *IN VITRO* CULTURE SYSTEMS WITH INCREASED SENSITIVITY FOR DRUG-INDUCED CYTOTOXICITY – PAPER I

The mechanisms by which DILI develop are complex. Apart from a direct cytotoxic effect, drug induced liver injuries often involve the interplay between several different types of cells [13]. Although mono-cultures of primary hepatocytes or HepaRG cells are valuable tools for drug screening and drug induced hepatotoxicity, high metabolic capacity does not always correlate with high sensitivity [255]. There are a growing number of *in vivo* studies showing that inflammatory mediators play a central role in the idiosyncratic potential of certain drugs, Kupffer cells being particular important in these processes. The role of Kupffer cells in DILI has elegantly been shown in several animal studies where inactivation of Kupffer cells with gadolinium chloride prevents the hepatotoxic effect exerted by different drugs and toxins, such as paracetamol [240] and carbon tetrachloride [241]. The release of TNFα from activated Kupffer cells has also been shown to be a key factor in the progression of alcoholic-induced liver disease in rats [256, 257].

4.1.1 Co-culture set-up

Previously, co-culture systems of endotoxin-stimulated, non-parenchymal cells together with hepatocytes from rat were shown to sensitize the hepatocytes and affect the hepatic protein synthesis, mediated via soluble factors produced by the non-parenchymal cells [233, 258]. In order to evaluate whether the introduction of inflammatory cells could increase the sensitivity for drug-induced cytotoxicity we developed an *in vitro* co-culture system [232] based on hepatocytes and monocytes. Animal studies have poor predictively of DILI in man, thus a human *in vitro* system was desirable. Since no Kupffer cell lines were available at the time of the initiation of the study the human monocytic cell line THP-1 was used and co-cultured together with the human hepatoma cell line Huh7. As model drugs a pair of PPARγ agonists, troglitazone and rosiglitazone, were used. Troglitazone was withdrawn from the market in 2000 due to idiosyncratic hepatocellular hepatotoxicity in patients, whereas rosiglitazone is generally not considered to cause liver insult [212]. A concentration of troglitazone that caused modest cytotoxicity was chosen based on dose titration evaluation in single cultures. Three different variants of the co-culture system were evaluated: a model where the two cell types were cultured in direct contact with each other, a model where the cells was separated by a thin layer of collagen and an insert model where cells were separated by a thin porous membrane. The insert model (Figure 5) [232] was found to yield the highest sensitivity with respect to cell viability and the expression of inflammatory mediators and was therefore chosen for the study.

4.1.2 Increased sensitivity for troglitazone-induced toxicity in the co-culture system

Troglitazone treatment resulted in a modest cytotoxic effect in the single cultures but the toxic response was significantly potentiated in the co-culture with the viability

decreasing to about 40% for both cell types. Moreover, the toxic onset was generally faster in co-cultures compared to single cultures and was especially evident for the THP-1 cells, suggesting a synergistic cytotoxic effect by soluble mediators released between the cells. An initial screening experiment of the gene expression in troglitazone and rosiglitazone treated co-cultures identified several stress related pathways that was analyzed more in depth. RT-PCR analysis showed that troglitazone treatment induced several stress related, such as metallothionein 2A (MT2A), DNA damage-inducible transcript 3 (DDIT3), the chemokines CXCL2 and CXCL10 and heat shock protein A6 (HspA6), in the Huh7 cells from the co-culture compared to the Huh7 treated in single culture. Similar results were observed regarding DDIT3, MT2A, CXCL2 and CXCL10 in troglitazone treated THP-1 cells in co-culture compared to the THP-1 single cultures. Moreover, troglitazone treatment also induced the expression of IL-1β in the THP-1 cells from the co-culture. Contrary, the gene expression of TNFα and IL-6 in THP-1 cells was significantly higher in single cultures compared to co-cultures after troglitazone treatment. This suggests that the hepatocytes may have a modulatory effect on the responses of the non-parenchymal cells in this model, as supported by a study by Steinhorn *et al.* where they show that the LPS-stimulated secretion of IL-6 by rat non-parenchymal cells was decreased in co-cultures with hepatocytes [259]. In contrast to troglitazone, no toxic effects were detected in either cell type in the rosiglitazone treated co-cultures and single cultures.

4.1.3 The hepatotoxic mechanism of troglitazone

Several different mechanisms are suggested to cause the idiosyncratic effects observed in troglitazone treated patients. Troglitazone has been shown to induce apoptosis in hepatocytes and has been strongly associated to mitochondrial dysfunction caused by decreased ATP levels, mitochondrial DNA damage, loss of mitochondrial potential and increased levels of reactive oxygen species (ROS) [242, 260-262]. Indeed, DDIT3 expression, known to be regulated by mitochondrial oxidative stress, was up-regulated in our troglitazone treated cells [263] which is supported by a study in primary hepatocytes showing that DDIT3 expression is increased after troglitazone treatment [264]. The troglitazone-induced mRNA expression of MT2A, known to be affected by oxidative stress due to mitochondrial deficiencies, was also observed in both cell types of the co-culture system [265]. Mitochondrial Hsp60 (heat shock protein 60) has both pro-and anti-apoptotic properties and may promote caspase 3 signaling [266]. In line with the ROS theory, a paper by Rachek *et al.* showed that the troglitazone-induced toxicity could be reduced by antioxidants [242]. In our cells a significant increase in catalase gene expression was seen in Huh7 cells in response to both PPARγ-agonists and was generally higher expressed in the co-cultures compared to the single cultures. Treatment with antioxidants, such as catalase and Trolox, only had low effects on the troglitazone-induced toxicity in our cells indicating that ROS only play a minor role. However, there are reports that suggest that traditional antioxidants may not be sufficient to protect against mitochondrial damage [267].

Troglitazone is mainly metabolized to sulfate and glucuronide conjugates which are not considered to be hepatotoxic, however, reactive metabolites have also been found in patients treated with troglitazone [212]. Troglitazone has been suggested to be

metabolized by CYP3A4 and to a lesser extent by CYP2C8. In addition, troglitazone has shown to be a potent inducer of CYP3A4 [212, 268]. In our system troglitazone appears to cause a direct toxic effect as neither of the cell systems showed any extensive metabolism of the drug. This is supported by *in vitro* studies where it has been concluded that troglitazone induces toxicity by itself rater that the reactive metabolites generated [262, 269, 270]. Moreover, studies in primary hepatocytes show that the mitochondrial dysfunction preceded the formation of troglitazone metabolites [269].

A immune mediated reactions has also been suggested to play a role for the toxic effects caused by troglitazone [271], possibly a response to the apoptotic and necrotic cell death seen [212]. The induced expression of the chemokines CXCL2 and CXCL10 detected in the co-cultures indicate that there is an ongoing production of inflammatory mediators that may very well be able to cause infiltration of immune cells like neutrophils and monocytes *in vivo*, thereby potentiating the inflammatory response [41, 272, 273]. When treated with troglitazone the THP-1 cells in single culture exhibited an increased expression of TNFα. IL-6 was induced in the THP-1 cells in both single and co-cultures, but predominately in single cultures. IL-1β, on the other hand, was mainly induced in the co-cultures. All three cytokines are known to promote an inflammatory response [274, 275], but TNFα and IL-6 are also known to have a hepatoprotective role [40, 51]. There are several studies that report that pro-inflammatory cytokines are not induced after troglitazone treatment [276, 277]. The overall increase in pro-inflammatory cytokines in our study is modest but is generally more pronounced in the co-culture and support the viability data. Our results suggest interplay between the two cell types, adding to the toxic insult. Whether these mediators play a role in the troglitazone-induced insult or whether the observed toxicity is a combination of other mechanisms warrants further investigation. Further development of this co-culture model requires the use of metabolically competent cells and the incorporation of other cell types known to be important for inflammatory mediated responses, such as endothelial cells [60] and stellate cells [49]. In addition, a 3D culture system might improve long term functional maintenance of these cells.

We are all constantly exposed to bacterial endotoxin, such as LPS (lipopolysaccharides), an important pro-inflammatory agent that can trigger the inflammatory innate immune response [40]. Dietary factors, such as alcohol and a high fat diet, significantly increase the translocation of LPS from the gut into the blood stream and may increase the sensitivity of the liver to toxic insults [278, 279]. Co-administration of hepatotoxins like chlorpromazine [280] and ethanol [281] together with a non-hepatotoxic dose of LPS has shown to prime the Kupffer cells and potentiate the hepatic insult of these substances *in vivo*, even when given in non-toxic doses. A similar approach has been used *in vitro* where a rat co-culture model with Kupffer and hepatic parenchymal cells was more sensitive to hepatotoxic substances after LPS activation of the Kupffer cells [233]. Application of this two-hit approach might further improve the sensitivity of our co-culture system.

4.2 HEPATOCYTE-LIKE CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS DIFFERNTIATED VIA DEFINITIVE ENDODERM – PAPERS II AND IV

4.2.1 Directed differentiation of human embryonic stem cells in two-dimensional culture – paper II

The first article of hepatocyte-like cells generated from human embryonic stem cells (hESC) came out only a few years after the first successful generation of hESC cultures [282]. Since then several research groups have developed protocols for hepatic differentiation of hESCs, largely based on what is known about the developmental pathway of the liver during embryogenesis [187, 189-193, 283]. Unwanted spontaneous differentiation has been a major problem which has increased the focus on more directed and controlled differentiation of hESC with the aim to generate more homogenous cell populations of hepatocyte-like cells. The liver originates from the definitive endoderm (DE) and the induction and fate of these cells is regulated by a complex integrated network of gene regulators [17, 20]. In paper II, we applied a directed, multi-stage protocol where hESCs were differentiated towards hepatocyte-like cells, via DE, under conditions known to support the hepatic development *in vivo* (Figure 8).

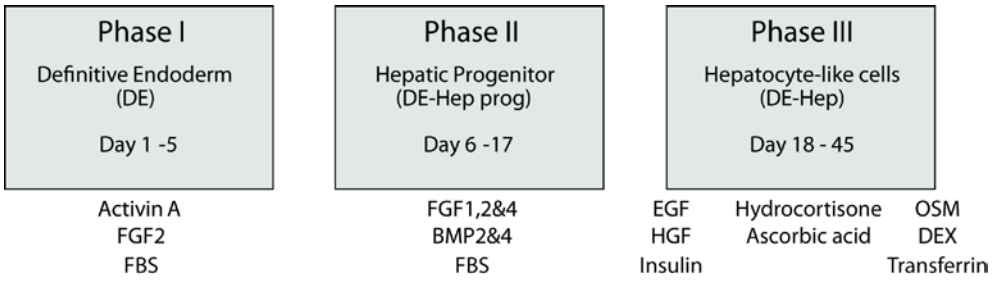

Figure 8. Schematic overview of the differentiation strategy of hESC into definitive endoderm derived hepatocytes. The differentiation protocol is divided into three main phases. The starting material consists of undifferentiated hESC cultured on mouse embryonic fibroblasts (MEF). In phase I the hESC are induced into definitive endoderm (DE). In phase II the DE is induced to liver progenitor cells (DE-Hep prog). During phase III the cells are maturated to hepatocyte-like cells (DE-Hep). Adapted from paper II.

During embryogenesis a primitive endoderm (PrE) is also formed, giving rise to an extra-embryonic endoderm [284]. In the early stage of embryogenesis DE and PrE have a very similar differentiation pattern, despite their different faiths [285], with only a few known markers to discriminate between the two [286, 287]. In order to validate our protocol we compared the DE differentiated hESCs to intrinsic (spontaneous) differentiated (ID) hESCs cultured in VitroHES™ supplemented with FGF2. The ID protocol has previously been characterized and has been shown to generate hepatocyte-like cells with protein expression of GSTs [288] and mRNA expression of CYPs [289]. However, the ID protocol gives rise to a mixed cell population [289] and is considered to mainly mediate differentiation into PrE cells [287]. The DE cells were also compared to HepG2 cells and primary hepatocytes.

In the first phase of the differentiation protocol, Activin A was added to mimic endogenous Nodal signaling [16, 189], known to be particularly important for DE formation [16, 19]. A low concentration of serum (0.2%) was also added to block phosphatidylinositol 3-kinase (PI3K) activity since it has been shown to inhibit the Activin A induced differentiation of hESC [290]. Other publications have shown that WNT3A also contribute to an accelerated and improved hESC differentiation towards DE [190, 291], however, in our system Activin A in combination with FGF2 proved to be superior. A high yield of morphological changed DE cells was generated, characterized by the expression of SOX17, FOXA2 and CXCR4 [189], as compared to the ID cells. Only a minor part of the DE cells expressed the PrE marker SOX7 [23]. Additionally, the DE cells did not show the same elevated levels of early liver markers, like α-fetoprotein (AFP), as the intrinsic cells did which at this early stage in development would indicate the presence of PrE cells.

In phase II, treatment with a combination of BMPs and FGFs, mimicking the interaction with and stimuli by mesoderm tissues observed *in vivo* [25, 292], changed the morphology of the cells into polygonal shaped cells and induced the expression of early hepatocyte specific genes like HNF3β, HNF4α, and cytokeratin 8 and 18 (CK8 and CK18). Moreover, the cells showed signs of early liver development by significantly elevated expression of A1AT (α1-antitrypsin), AFP and FABP1 (fatty acid-binding protein 1), compared to the intrinsically differentiated control cells. Intriguingly, some cells co-expressed EpCAM (epithelial cell adhesion molecule) and CK19 which suggests the presence of hepatoblasts, a common progenitor to the hepatocytes and cholangiocyte, confirming early liver development [227].

Hepatocyte-like cells (DE-Hep) were generated in Phase III by the addition of various maturation factors, such as Oncostatin M (OSM) and dexamethasone (DEX), important for liver formation and hepatocyte maturation and function [20, 26, 27, 29]. The DE-Hep cells had typical polygonal-shaped hepatocyte morphology with several bi-nuclear cells, clearly different from the cell morphology of the intrinsically differentiated cells (Figure 9A and 9C, respectively). Interestingly, many of the DE-Hep cells arranged themselves in substructures of CYP3A4/7 positive cells that have not been reported elsewhere (Figure 9B).

Apart from the hepatocyte like morphology, the DE-Hep cells also showed significantly up-regulated mRNA expression of several liver related genes such as A1AT, albumin, AFP, HNF4α and transporters (MRP2 and OATP2), compared to the intrinsically differentiated control cells. Most of these genes were also detected at protein level. The DE-Hep possessed several hepatocyte functions, such as urea secretion, active indocyanine green (ICG) uptake (clearance test) and glycogen storage. The expression of catalytically active CYP enzymes is also an important marker for mature hepatocytes [111]. The mRNA expression of CYP1A2, 2C9 and 3A4 was significantly higher in DE-Hep cells compared to both the intrinsically differentiated cells and HepG2 cells. Protein expression of CYP1A2 and 3A4 was also confirmed. A functional drug metabolism test of the DE-Hep cultures at day 29 revealed that the DE-Hep cells were metabolically competent for CYP1A, CYP3A and CYP2C. To-conclude, the guided differentiation approach proved to be superior to intrinsic hESC

differentiation regarding hepatocyte-like morphology and hepatic protein expression. These data are supported by Synnergren *et al*. who showed major transcriptional differences between DE-differentiation and intrinsic differentiation when analyzed on a global scale [287]. However, apart from higher CYP1A2 activities observed in DE-Hep cells there was no significant difference in CYP activity when compared to the intrinsically differentiated cells. A possible reason, as suggested by a gene array analysis [287] could be that the intrinsic cultures may contain other CYP expressing cells like intestinal cells.

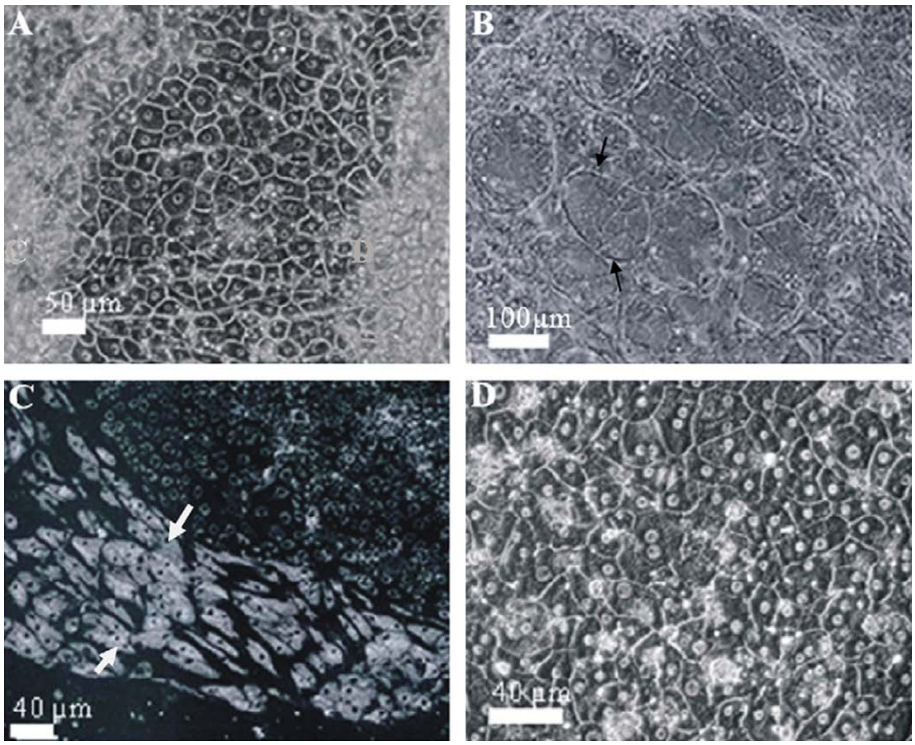

Figure 9. Phase-contrast images of DE-Hep in phase III and intrinsically differentiated (ID) cells. A) DE-Hep cultures at day 17. The cells exhibit a hepatocyte-like morphology and are frequently bi-nucleated. B) DE-Hep cultures at day 20 are starting to form multicellular structures of CYP3A4/7 positive cells (black arrows). C) Intrinsically differentiated control cells at day 23 (white arrows). The hepatocyte-like cells are located only at the periphery of the growing colony and show different morphology with no clear sub-structures compared to the DE-Hep cells. D) Human primary hepatocytes in culture. Adapted from paper II.

4.2.2 Directed differentiation of human embryonic stem cells in three-dimensional culture – paper IV

It is well known that primary hepatocytes depend on high cell density and well organized tissue architecture for the maintenance of their differentiated functions [218, 219]. This is nicely illustrated by our preliminary results on primary hepatocytes cultured in the perfused three-dimensional (3D) bioreactors. Primary hepatocytes cultured in 2 ml and 0.5 ml bioreactors showed maintained CYP activities up to two weeks (Figure 10).

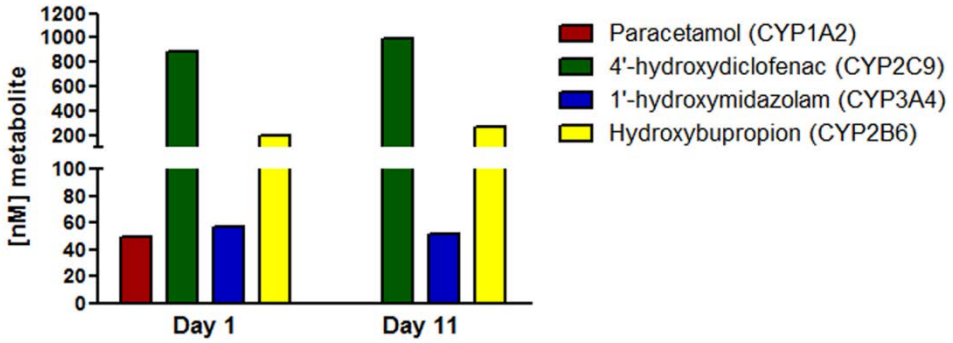

Figure 10. Metabolite formation after 10h incubation with four different CYP enzyme substrates measured at day 1 and day 11 in human primary hepatocytes cultured in a perfused bioreactor. Shown here is one representative experiment performed in a 0.5 ml bioreactor (Sivertsson, L., unpublished data).

Similar results have recently been published elsewhere, using the same bioreactor system, showing that the hepatocytes are able to form liver-like structures in the bioreactors [227, 228]. Specific inter-cellular interactions and cellular organization are also important aspects in embryonic liver development [17, 20]. Different 3D bioreactor cultures, both perfused [293] and stirred [294], has successfully been used for hESC expansion. A study by Baharvand *et al.* showed that differentiation of hESC in collagen scaffolds resulted in improved metabolic activity compared to 2D differentiated cells [295]. In a recent publication, larger perfused bioreactors (8 ml) were successfully used for hepatic differentiation of hESC [296] and maturation of fetal hepatocytes [297], indicating that the 3D perfused bioreactor system may be a valuable tool for the differentiation of stem cells into functional hepatocytes. In order to evaluate if culture in the 3D bioreactor system could improve the hESC differentiation process, we compared cells culture in the bioreactor to cells cultured in conventional 2D culture as described in paper II. The undifferentiated hESC (UD) were stepwise guided through hepatic development, via DE and hepatic progenitors (PRO), to hepatocyte-like cells (HEP) as described in paper II but with some changes in the protocol (Figure 11).

Originally, hESCs were cultured on top of a mouse fibroblast feeder layer and passaged manually twice a week to prevent spontaneous differentiation, which was both time and labor demanding. In addition, the use of animal cells is associated with risks such as pathogen and viral infections. Cellectis, Cellartis AB has recently developed a feeder-free culture system that can be fully automated for large-scale propagation of embryonic stem cells, able to generate the large amount of cells needed to fill up the 2 ml bioreactors (minimum of 25 million) for this study. Sodium butyrate and DMSO were included in this differentiation protocol as they both have been shown to promote hESC hepatic differentiation [282, 298, 299]. Moreover, DMSO has also been frequently used to maintain or induce differentiation of primary hepatic cells [300, 301] as well as hepatic cell lines [175]. Fetal bovine serum (FBS) was added in phase II. FBS contains several important factors important for cells in culture, like growth factors, minerals and trace elements, transport proteins and lipids, however, the incubation time with serum was decreased in this protocol as it has been shown to negatively affect the differentiation of hESC [189] as well as the function of primary

hepatocytes [302]. Serum-free conditions have also been shown to result in better CYP activities in hESC differentiated hepatocyte-like cells [303].

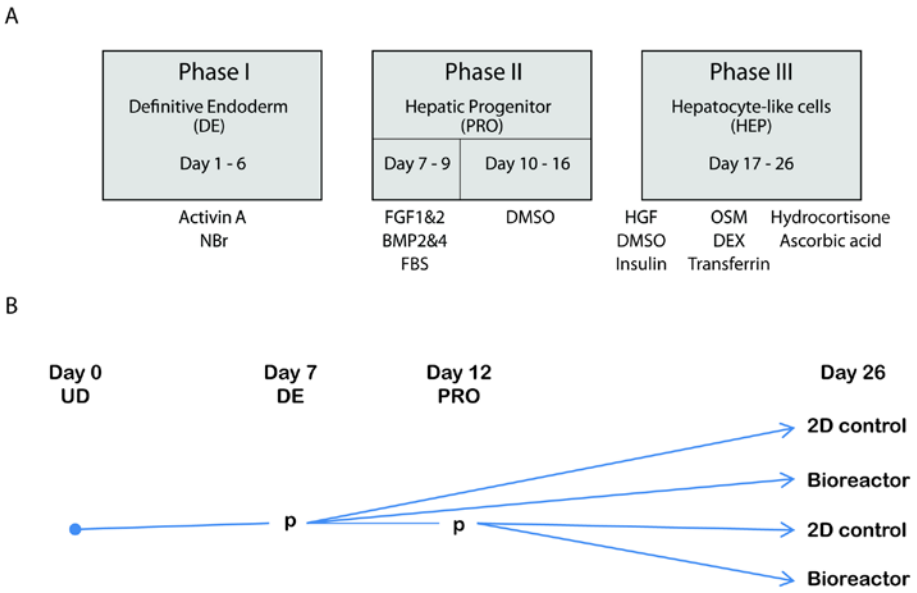

Figure 11. A) Schematic overview of the hepatic differentiation strategy of undifferentiated hESC cultured under feeder-free conditions. The differentiation protocol is divided into three main phases: phase I where the hESC are induced into definitive endoderm (DE), phase II where the DE is induced to liver progenitor cells (PRO) and phase III where the cells are maturated into liver-like cells (HEP). B) The cells were inoculated into the bioreactors either as definitive endoderm (DE) on day 7 or as hepatic progenitors (PRO) on day 12. The cells were passaged (p) and either inoculated into the 3D bioreactor or seeded in 2D controls. NBr: sodium butyrate. Adapted from paper IV.

During the first 7 days of differentiation the mRNA expression of the stem cell markers NANOG and OCT-4 dramatically decreased and in the PRO stage they were no longer detectable where, in contrast, the levels of the hepatic progenitor marker AFP drastically increased. A global transcript array analysis of HEPs revealed significant differences between 2D and 3D cultured cells. In total 102 annotated genes were differentially up-regulated and 63 genes were down-regulated ($p<0.05$ and $FC>2$) when the cells were cultured in the 3D system compared to the 2D system. The functional properties of the differentially expressed genes were evaluated by Gene Ontology enrichment analysis that showed that a large fraction of the enriched annotated genes were related to various metabolic processes. A KEGG pathways analysis identified 10 pathways, highly related to liver specific functions that were significantly up-regulated in cells differentiated in 3D. Contrary, pathways associated with apoptosis and cell proliferation were generally down regulated, as can usually be seen in terminally differentiated cells. An association analysis of the transcriptional profiles compared to human primary hepatocytes showed a clear pattern of stepwise hepatic maturation of hESC to DE, PRO and HEP (both in 2D and 3D), with the highest similarity for the 3D HEP cells. The expression of a large number of hepatic markers (ALB, TAT, FABP1), enzymes (CYP7A1, 1A2, 2C9, UGT2B7) and transporters (MRP2, OCT1) increased during the differentiation process were CYP7A1, CYP2C9 and CYP3A4 were significantly higher expressed in 3D HEP cells compared to 2D HEP cells.

Immunohistochemical staining of the HEP cells from the bioreactor showed that the cells successfully arranged themselves between the capillaries. The HEP cells stained positive for A1AT and CYP3A4 protein expression, suggesting a hepatocyte-like phenotype. The apical efflux transporter BCRP (Breast Cancer Resistance Protein) was also present in the bioreactor tissue. Regular analysis of metabolic parameters in the media circulating through the bioreactors indicated that the cells were metabolically active as they consumed glucose. When treated with a CYP substrate cocktail, the hepatocyte-like cells cultured in the bioreactor produced higher levels of paracetamol, hydroxybupropion and 1'-hydroxymidazolam metabolites compared to the 2D cultures, indicating higher CYP1A2, CYP2B6 and CYP3A4-mediated activities, respectively. However, the metabolic activity in the 3D differentiated HEP cells was considerably lower when compared to hPH.

Earlier studies have shown differentiation of hESC cultures in three-dimensional (3D) aggregates called embryoid bodies [304, 305]. This approach proved to be inefficient and resulted in a mixed population of all three germ layers [16, 29]. However, it nicely illustrates the influence that a 3D milieu has for hESC differentiation since the spontaneous differentiation seen in the embryoid bodies are far more complex than the simple spontaneous differentiation of hESC initiated by discontinuous passaging [304, 305]. To date, the most efficient differentiation protocols are those applying DE differentiation using soluble growth factors on hESC cultured in monolayers. However, in a static 2D culture, gas exchange only occurs via passive diffusion from the air to the media and media exchange is discontinuous. It has been shown that the oxygen concentration around the cells differs greatly comparing the *in vivo* with *in vitro* conditions and is mainly due to the low solubility of oxygen in cell culturing media [302]. The 3D bioreactor technology used here is a dynamic system with continuous media renewal, creating a homeostatic environment that mimics the capillary structures present *in vivo* [227]. Growth factors and nutrients are supplied and cell waste products are removed with low gradients. In addition, oxygen is continuously added in a low gradient manner, which is important since it has been shown that low oxygen supply affects hepatocyte metabolism [302].

The directed differentiation protocol presented here generates a high yield of differentiated hepatic cells that display many hepatocyte-like characteristics and functions. Moreover, our perfused 3D system supports and improves the directed hepatic differentiation and maturation of hESC. However, the generation of fully functional hepatocytes is still some way ahead and further research regarding the cellular and molecular mechanisms involved in this complex process is required. Apart from the media composition, other extracellular and environmental factors should be considered to take the differentiation process one step further. A few examples are incorporation of other cell types and/or the addition of extra cellular matrix (ECM) to the 3D cultures. In tissue, cells do not only attach to each other but also to the ECM which will affect how the cells respond to stimuli from their surroundings. Recently, Hay *et al.* studied the effect of ECM on hESC-derived hepatocyte-like cells and identified a polyurethane matrix that enhanced hESC-derived hepatocyte functionality and long-term growth [303]. In the future it should also be investigated whether

coating of the bioreactor and its fibers can improve the differentiation process of our cells. Paracrine signals from mesenchymal cells have also been shown to be important for the expansion and hepatic differentiation of human stem cells [278] as illustrated *in vitro* by a co-culture of mouse embryonic stem cells and mesoderm cells [306]. Differentiation of oval cells has been performed in 3D culture systems with mesenchymal feeder cells [307] and stellate cells [197] which facilitated their differentiation into mature hepatocytes. Incorporation of hESC or DE derivates together with primary liver cells in the bioreactor might be another way to generate mature hepatocytes. This approach has previously shown to be effective for differentiation of mouse embryonic stem cells, co-cultured together with human non-parenchymal liver cells [308]. Recently, interesting results have been published by a Japanese group which showed effective hepatic differentiation of hESC and iPSC by sequential transduction with various transcription factors, such as FOXA2, HNF4α and HNF1α [202, 309]. Notably, hepatocyte-like cells have also been generated directly from murine fibroblasts by transduction with the same factors, without the need for cellular pluripotency [310, 311].

4.3 HIGH CELL DENSITY-INHIBITED PROLIFERATION INDUCES CYP3A4 CATALYTIC ACTIVITY IN HUH7 CELLS POSSIBLY REGULATED BY CDK2 MEDIATED PXR ACTIVATION – PAPERS III AND V

Primary hepatocytes are highly malleable, an important feature during liver injury when the cells exhibit a remarkable regenerating capacity via proliferation [46]. However, these properties constitute a problem when the cells are used for research purposes. When isolated from their microenvironment and placed in 2D cultures primary hepatocytes rapidly de-differentiate into a population of adult liver progenitors [167], exhibiting an extensive loss in many mature liver function such as CYP enzyme activities [133, 168]. The decline was been shown to been the most rapid for CYP3A4 [312]. One of the early mechanistic changes observed during isolation of hepatocytes is changed levels of transcription factors where many liver-enriched liver transcription factors are rapidly down-regulated [106]. The mechanism behind this phenomenon is not well understood but has been suggested to be caused by stress signaling induced during the isolation process and a loss of cellular ultrastructure [313, 314]. Primary hepatocytes are highly dependent on high cell density and tight cell-cell contacts, not only for the maintenance of their differentiated functions and organized tissue architecture, but also for the regulation of their proliferation [218, 219]. Previously, it has been shown that confluent culture of human hepatoma cell lines BC2 and B16A2 cause the cells to re-differentiate, resulting in a gene expression pattern that is more similar to primary hepatocytes [315, 316]. Thus, we conducted an in-depth analysis of the capability of the human hepatoma cell line Huh7 to re-differentiate to more functional hepatic cells when grown under confluent conditions.

4.3.1 Confluent culture results in spontaneous differentiation and increased CYP3A4 catalytic activity

The Huh7 cells were cultured confluent up to 5 weeks and compared to HepG2 cells cultured under the same conditions, to sub-confluent Huh7 control cells and to human liver tissue (HL). Growing the Huh7 cells confluent generated a dense monolayer of cells with a lower nuclear-to-cytoplasm ratio and the cells contained large distinct nucleoli, all suggestive of a more hepatocyte morphology. A whole transcriptome analysis with a combined criterion of $p< 0.05$ and FC >2 was used to analyze statistical and biological differences between 4 weeks confluent and subconfluent control cells. Transcripts from pathways related to cell proliferation were decreased and the cells achieved a more hepatocyte-like phenotype with improved liver specific functions, such as metabolism, when grown confluent (paper V). A more in depth TaqMan gene expression analysis revealed that confluent culture of Huh7 cells up to 5 weeks resulted in a gradual increase in mRNA expression levels for several CYPs, UGTs, transporters, transcription factors and other liver specific genes (paper III). The largest effect was observed for CYP3A4 with a substantial increase in mRNA and protein expression after 4 weeks of confluent culture, to levels comparable to that seen in human liver. However, no effect was observed on the expression levels of the other CYP3A isoforms. The increase in CYP3A4 mRNA and protein expression was accompanied by a time-dependent increase in catalytic activity that correlated well with the protein levels, determined to be approximately 30% of the activity in HL microsomes (paper III). The increase in CYP3A4 activity was also accompanied by increased protein levels of the electron donors NADPH cytochrome P450 reductase (POR) and cytochrome b_5. In addition, POR enzyme activity was increased to approximately 50% of that observed in HL. The 4 week confluent Huh7 cells were also able to metabolize testosterone and midazolam, confirming the previous activity result. Since the confluent cells were metabolically active, they were also able to respond to CYP3A4-mediated aflatoxin B1 cytotoxicity. Moreover, the toxic effect was effectively prevented by the CYP3A4 inhibitor ketoconazole (paper III). These results show that high cell density and tight cell-cell contacts are indeed important for the functionality of the Huh7 cells, much like the situation in the liver *in vivo*. The importance of cell density and cell-cell contacts was further underscored when the CYP3A4 competent confluent cells were trypsinated and replated subconfluent, and the CYP3A4 levels rapidly dropped back to those observed in the subconfluent control cells (paper V). This resembles the *in vivo* situation where CYP3A4 expression drastically decreases during liver regeneration [317]. The Huh7 cells were able to maintain CYP3A4 activities in long-time culture, albeit at lower levels. After 8 weeks of confluence the activity levels were comparable to the levels seen in 3 weeks confluent cells.

4.3.2 Cell line specific differentiation during confluent culture

It is clear that the phenotypical change occurring during confluent growth is cell line specific and does not apply to all hepatic cell lines, as illustrated by our results on HepG2 cells. Very little effect was seen in HepG2 cells cultured under the same conditions as the Huh7 cells, with no effect on any of the CYP genes investigated

(paper III). The reason behind the inability of HepG2 to re-differentiate is not known, but probably is related to the proliferation status of HepG2 cells which seems to not be contact inhibited, contrary to the Huh7 cells. Confluent growth of the B16A2 hepatoma cell line also resulted in an increase in metabolic pathways and liver specific genes, however, these cells did not express the same levels of metabolically competent genes as the Huh7 cells. In B16A2 cells only a moderate effect was seen for CYP3A4 (about 8-fold increase) compared to the massive effect in the Huh7 cells and might be caused by the lack of PXR and CAR expression in B16A2 cells [316]. Contrary, the B16A2 cells showed increased expression in CYP2E1 (about 7-fold) [316] which was unaffected in the Huh7 cells.

4.3.3 Transcriptional regulation of CYP3A4

In order to investigate if the increased *CYP3A4* gene expression in the confluent Huh7 cells was indeed transcriptionally regulated, several reporter constructs containing the three well-known CYP3A4 5'-flanking regulatory regions, PROX, XREM and CLEM4, were created. The activities of all the different constructs were significantly higher in confluent cells compared to control cells and the largest activity was observed with the CYP3A4-PXC-luc construct (Figure 7, paper V), indicating that all three regions are important for CYP3A4 regulation in these cells. The transcriptional activity of the CYP3A4-PXC-luc was increased in a time-dependent manner during confluency and correlated well with the observed increases in CYP3A4 mRNA, protein and catalytic activity (paper V). Next the regulatory mechanisms underlying the CYP3A4 specific up-regulation were investigated. In confluent Huh7 cells, the mRNA expression levels of many of the hepatic transcription factors analyzed where similar to the levels observed in HL (paper III). The expression of CYP3A4 is known to be regulated by many different transcription factors with PXR considered to be the most important modulator [126-128]. Due to the low expression of PXR in many cell lines, most studies regarding PXR regulation have relied on transient transfection of PXR together with responsive reporter plasmids and often with the addition of inducers, such as rifampicin. In the Huh7 cells PXR mRNA and protein expression was increased during confluence and, moreover, PXR protein accumulated in the nuclei (paper V). This effect was endogenous and did not require any addition of inducers. Apart from the increased CYP3A4 expression, other PXR regulated CYPs, like CYP2B6 or CYP2C9 [145, 318], were also induced in the confluent cells. Furthermore, the addition of the PXR dependent CYP3A4 inducer rifampicin [158] significantly induced the CYP3A4 promoter activity as well as the CYP3A4 metabolic capacity in the confluent cells, confirming a regulatory role of PXR in our confluent cells. Contrary, rifampicin was unable to induce CYP3A4 expression in the subconfluent control cells, indicating that PXR is not transcriptionally competent in those cells. We could also conclude that endogenous RXR levels were also not a limiting factor in control cells (paper V). The CYP3A4 activity in the confluent cells was effectively inhibited by ketoconazole (paper III). PXR knock-down in confluent cells significantly reduced the PXR mRNA expression by 60% along with a 40% reduction of CYP3A4 mRNA expression compared to cells transfected with nontargeting siRNAs, further confirming the transcriptional role of PXR. Notably, when the confluent cells were trypsinated and replated subconfluent, the PXR protein levels rapidly dropped to levels

comparable to those observed in control cells (paper V). Thus, the effect of confluence on the PXR levels is reversible and follows the CYP3A4 expression data. Together these results indicate that the observed increase in CYP3A4 mRNA, protein and activity levels is, at least partly, mediated through transcriptional activation via the PXR receptor.

4.3.4 Cell division and PXR regulation

PXR is dynamically regulated by its phosphorylation status which in turn affects its expression levels, ligand and DNA binding, localization, interaction with other modulators and protein stability [319]. Several studies have demonstrated a role of phosphorylation-dependent signaling events in the regulation of PXR-mediated *CYP* gene expression [138, 139, 160] and several putative serine and threonine phosphorylation sites have been identified within the human PXR protein [138]. It has been shown that CDK2 (cyclin-dependent kinase 2) is able to inhibit PXR activity by directly phosphorylating the receptor at Ser350 [320]. Active CDK2 inhibits the interaction of human PXR with SRC-1 via phosphorylation of PXR which attenuates PXR-mediated *CYP3A4* gene expression [318, 320]. These findings were supported by another study in HepG2 cells, showing that the use of an antimitogenic factor inhibiting CDK2 or CDK2 knock-down resulted in increased levels of CYP3A4 [321]. CDK2 is a well-known key regulator of cell cycle progression. During liver regeneration, CDK2 is activated by CDK-activating kinase (CAK) and by association with the regulatory units cyclin E/A [318, 322]. The negative regulation of CDK2 has been shown to involve PP2Cs (protein phosphatase type 2C), which dephosphorylates and inactivate CDK2. As a result, cell cycle is arrested and the inhibitory effect of CDK2 is abolished, enhancing the hPXR-mediated *CYP3A4* promoter activity [318, 322]. We addressed whether CDK2 was involved in the regulation of CYP3A4 expression in the proliferation-inhibited confluent cells. Indeed, we found that the CDK2 protein expression was significantly higher in subconfluent control cells compared to confluent cells. Knocking-down CDK2 in proliferating control cells resulted in increased PXR and CYP3A4 protein levels. Moreover, when the confluent cells were trypsinated and replated at low density the cells started proliferating again, resulting in increased CDK2 levels and, as a consequence, decreased the PXR and CYP3A4 levels (paper V). Our results presented here are consistent with the *in vivo* concept where both the activity of hPXR and the expression of CYP3A4 are regulated in a cell cycle- and cell proliferation-dependent manner (Figure 12) [317, 318].

4.3.5 DMSO effect on cell differentiation

DMSO is a common solvent used in drug metabolism and toxicity studies but is also used extensively to induce and maintain hepatic functions of primary cells [300, 301], oval cells [323], stem cells [299] and cell lines [175]. Moreover, DMSO has been used to induce hepatic functions in Huh7 cells where culture in 1% DMSO stops the proliferation and generate cells with more hepatocyte-like phenotypes and induced expression of several liver specific genes and phase I and II enzymes [324]. Choi *et al.* also describe a drastic effect on CYP3A4 expression when culturing the cells in DMSO for several weeks [324], comparable to our results in the confluent cells. However, we

could demonstrate that growing Huh7 cells confluent for 4 weeks in the presence of 1% DMSO did not have any additional effect on CYP3A4 protein and activity levels as compared with 4-week confluent cells cultured without DMSO (paper III). In the data presented by Choi *et al.*, DMSO treatment resulted in increased levels of CAR whereas PXR was unaffected, moreover, neither of the two transcription factors was inducible [324]. This suggests major differences in the regulatory mechanism behind the CYP3A4 induction in these two cell systems. In addition, it has been shown that DMSO inhibits several P450s, as well as CYP3A4, in a substrate-dependent way and would therefore not provide inaccurate assessment of drug interaction potentials [325, 326].

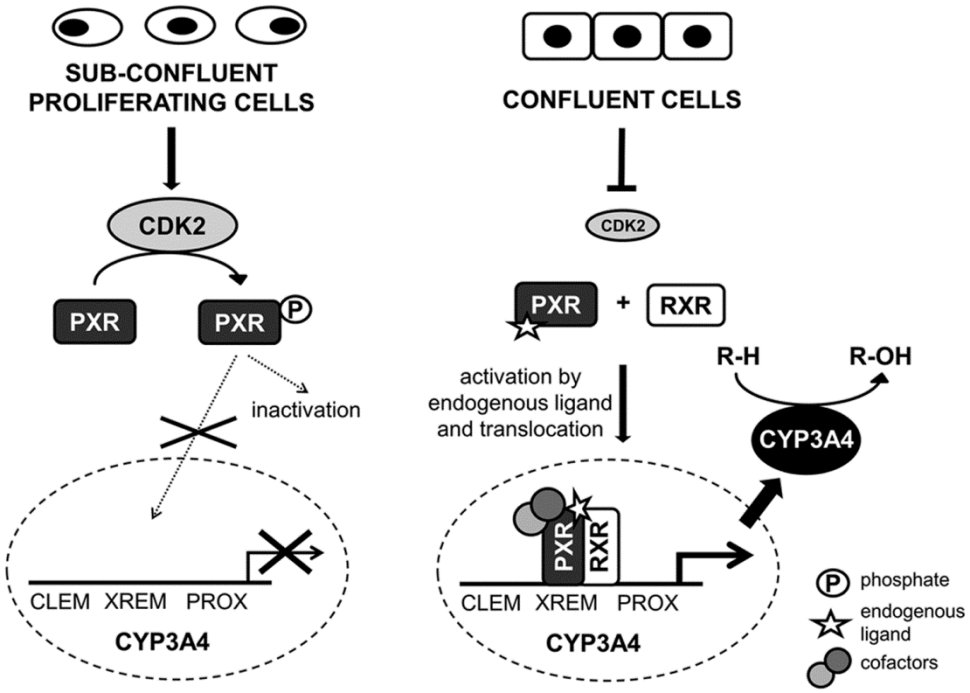

Figure 12. Proposed mechanism for the involvement of CDK2 in the PXR dependent regulation of CYP3A4 in Huh7 cells. In the non-confluent proliferating Huh7 cells the levels of active CDK2 are increased, which results in increased levels of phosphorylated PXR. Phosphorylation of PXR affects ligand binding and cytosol-nucleus translocation. Phosphorylation may also target PXR to be degraded and, subsequently, the transcriptional activation of CYP3A4 is reduced. In the confluent cells, cellular proliferation is inhibited and cell cycle is arrested, resulting in decreased levels of active CDK2. Unphosphorylated PXR can now be activated, possibly by an endogenous ligand or by other processes, heterodimerize with RXR and be translocated into the nucleus. Here it can bind, together with cofactors, to the regulatory elements of the *CYP3A4* gene, resulting in increased expression of CYP3A4. Figure from paper V.

The mechanism by which DMSO induces differentiation of certain cell types still remains obscure but DMSO has been shown to increase the levels of PKC [327], to inhibit histone deacetylases [328] and to increase the levels of cadherin and integrin complexes [329], all contributing to inhibited proliferation and increased gene transcription. Our hypothesis behind the altered differentiation status in our cells relies on a similar mechanism, but is induced by the formation of endogenous cell-cell contacts rather than by chemicals. Cell-cell contacts via adherens junctions (E-

cadherin/β-catenin) are essential for terminal differentiation of hepatocytes [330]. Immunocytochemical staining of confluent Huh7 cells indicate that the cells formed both tight junctions (coxsackie virus and adenovirus receptor staining, CXADR) and adherens junctions (β-catenin staining), both important for proper hepatocyte polarization and function (Figure 13).

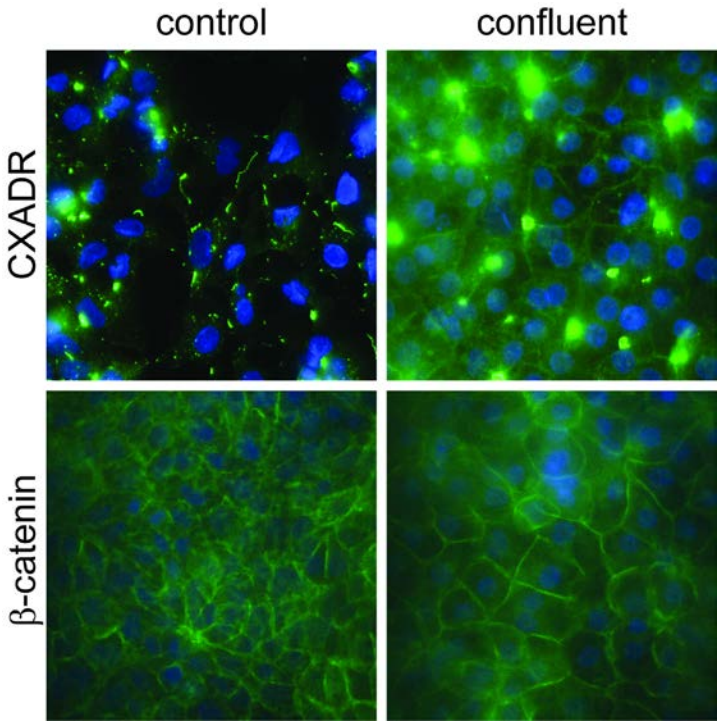

Figure 13. Immunocytochemical staining with CXADR (coxsackie virus and adenovirus receptor) and β-catenin, representative of tight junctions and adherens junction formation respectively (Sivertsson, L., unpublished data).

In the subconfluent control cells, only the places were the cells touch each other have positive CXADR staining whereas in the confluent cells homogenous tight junctions have been formed between the cells. The highly intense CXADR staining on certain locations between the confluent cells may be indicative of bile canalicular structures. Positive β-catenin staining is seen in the confluent cells, indicative of the formation of adherens junctions. The control cells, which have just reached confluence, also stain positive for β-catenin although with a more diffuse staining pattern (Figure 14). These results are in good agreement with the array results and support cell-cell contact inhibited proliferation in the confluent cells.

In summary, as the Huh7 cells grow confluent proliferation is inhibited and the cells undergo a spontaneous differentiation without the addition of inducers. CYP3A4 is clinically the most important CYP enzyme, estimated to be involved in the metabolism of about 50% of the marketed drugs [111]. Better understanding of the regulation of *CYP3A4* and the encoded enzyme is therefore of major clinical significance. Moreover, drugs interacting with PXR are considered likely to be involved in drug-drug interactions. It is well accepted that PXR regulation exhibits a species-specific profile which makes the confluent Huh7 cells a valuable system to assess human PXR

activation as well as drug-drug interactions involving PXR and CYP3A4. Moreover, contrary to transient transfection models, involving plasmid based PXR and CYP3A4 promoter constructs, the actual effect on the metabolic CYP3A4 activity can be assessed. The high constitutive expression of CYP3A4 also makes the confluent Huh7 cell system useful to study mechanisms behind *PXR* and *CYP3A4* gene regulation and might contribute to a better understanding of the large inter-individual differences in hepatic CYP3A4 expression.

5 CONCLUSION

This thesis can be concluded as follows:

- Treatment of a human *in vitro* co-culture cell model, consisting of hepatocytes and monocytes, with the hepatotoxic drug troglitazone results in a potentiated and more rapid cytotoxic response compared to single cell cultures. Troglitazone treatment also causes increased expression of several inflammatory and stress-related genes in both cell types during co-culture. In contrast, the non-hepatotoxic drug rosiglitazone does not cause any significantly effect in either cell systems.

- Our controlled, stepwise differentiation protocol guides the human embryonic stem cells, via definitive endoderm and progenitor stages, to hepatocyte-like cells. Gene and protein expression as well as functional data, indicate that the hepatocyte-like cells exhibit many hepatocyte-specific features and functions, including CYP metabolic activities.

- The hepatic gene and protein expression and functionality of the differentiated hepatocyte-like cells are further improved by three-dimensional culture in the bioreactor system compared to the standard two-dimensional culture.

- Confluent culture of the human hepatoma cell line Huh7 induces a spontaneous hepatic differentiation process without the need for the addition of inducers. A major effect is observed for CYP3A4 gene and protein expression as well as catalytic activity.

- The large increase in CYP3A4 expression in the confluent Huh7 cells is mediated by increased PXR transcriptional activity, possibly as a result of decreased CDK2 activity and cell cycle arrest.

6 GENERAL SUMMARY AND FUTURE PERSPECTIVES

Drug induced liver injury (DILI) is a significant problem in drug discovery, much owing to the shortcomings of pre-clinical model systems which often fail in the extrapolation to human biology. The underlying mechanism by which DILI develops is complex and involves multicellular interactions [13]. Toxins affect not only the hepatocytes but also other cells, at concentrations that may not be toxic for the hepatocytes. Therefore, *in vitro* cell models ideally should include biotransformation-competent hepatocytes as well as other hepatic cell types, in order to accurately predict *in vivo* toxicity. A more *in vivo*-like milieu in 3D cultures with different cell types has shown to be crucial for the maintenance of hepatocyte specific functions [229, 231], as well as for the facilitation of hepatic maturation of stem cells [20, 197, 307]. Apart from the hollow fiber bioreactor described in this thesis [227, 228], several promising 3D spheroid systems have recently been developed. Stir-tank reactors where hepatocyte spheroids are cultured under stirred, perfused, controlled conditions have shown maintained gene expression of phase I and phase II enzymes as well as hepatic functions, such as albumin and urea synthesis, up to two weeks [224]. InSphero (www.inspero.com) has developed human liver microtissue spheroids, which display long-term maintained liver functions, like albumin secretion and CYP activities. These spheroids contain both hepatocytes and non-parenchymal cells, including Kupffer cells, and have shown increased sensitivity to inflammation-mediated drug toxicity (InSphero). Interestingly, liver-like structures, e.g. bile canaliculi, are formed in the hollow fiber bioreactors [227, 228] and also in the spheroid systems [224]. Hence, these systems might be an important tool for the identification and characterization of drug induced pathological changes, like indications of cholestasis, necrosis and steatosis.

It has become evident that both metabolic as well as immune related factors are important for both type I and type II ADRs caused by many drugs [13]. To be able to predict immune-mediated drug toxicity, multi-cellular hepatic *in vitro* systems containing a population of immune competent cells could be of great importance [233]. Moreover, mixed-cellular systems could also be valuable “two-hit models”, where the hepatotoxic effects of drugs in combination with a sensitized liver state, such as steatosis and/or inflammatory stimuli like LPS, could be evaluated [331, 332]. Ideally, combined pairs of drugs with the same pharmacological target but different toxic potential (i.e. troglitazone/rosiglitazone) should be used to validate these *in vitro* systems. In addition, these systems might also assist in the search for more accurate and sensitive DILI biomarkers [13].

The unique characteristics of stem cells make them an attractive large-scale source of hepatic cells for drug development and safety assessment. Stem cells also have the potential to revolutionize the medical care regarding therapeutic applications. Liver transplantation treatments are often hampered due to shortage of donor organs and are also connected with high risks and lifelong immunosuppressive therapy for the patient [333]. As a complement, hepatocyte transplantation has successfully been applied in the clinic for bridging patients until liver recovery and also for treatment of patients with metabolic disorders [334, 335]. However, the shortage of high quality livers for

cell isolation highlights the need for hESC derived hepatocytes. Furthermore, the recent advances in human iPSC research might open up for individual-specific research and therapy by the creation of patient- and disease-specific stem cells [178]. By generating different cell types originating from the same patient and combining these into integrated artificial liver systems, the mechanistic differences between DILI sensitive and DILI insensitive patients may be studied. Moreover, the use of iPSC minimizes the risk of immune rejections as they are derived from the same individual. Moreover, these cells are not associated to the same ethical issues as hESC.

Even though good progress has been made in the last 15 years, the *in vitro* differentiation of hESC into linage-restricted functional cells has proven to be a challenging task. The use of stem cells for the generation of hepatocytes has yet to overcome several obstacles, like low metabolic functions, in order to fulfill the requirement as a powerful tool for drug development and safety assessment. The molecular program that initiates and sustains human liver development still remains elusive and we have to increase our understanding of the coordinated developmental cues/signals these cells receive *in vivo*. Furthermore, during liver development, hepatocytes mature in a 3D environment with a number of other cell types that are important for their differentiation. While the 3D hollow fiber bioreactors enable a more *in vivo* like environment, the technique has to be further developed and the incorporation of ECM and other cell types has to be evaluated. By combining several bioreactors in the same perfusion circuit it is possible to include bioreactors of hESC together with reactors containing other cell types, such as mesodermal cells or primary cells, so that the impact of soluble factors released by these cells can be evaluated. A similar set up could be applied for primary hepatocytes and immune cells with the aim to provide an immune sensitized system with long term metabolically competent hepatocytes for DILI studies.

By combining our knowledge regarding mechanisms and signaling pathways involved in liver regeneration, de-differentiation of isolated hepatocytes, maturation of semi-specialized cells like oval cells and hepatoblasts and hESC differentiation, we should get a better insight into the mechanisms behind hepatic cell specialization and how to prevent the loss of it. Moreover, the re-differentiation process of hepatoma cells, like the Huh7 cells, might also provide important clues as cancer cells and stem cells share many common features and high similarities in their overall gene expression patterns, miRNA expression and epigenetic status [336]. Several genes that frequently are up-regulated in tumors, such as c-myc [337], KLF4 [338] and β-catenin [17, 339], are also known to contribute to the hESC phenotype. Many of these genes also constitute the foundation of the successful generation of iPSC [178].

To conclude, in order to reduce the prevalence of ADRs and late stage drug discovery failures, more sophisticated human *in vitro* models have to be developed where cells are cultured under more physiological relevant conditions. At the same time, more effective and accurate *in vitro* screening methods of new drug candidates may also reduce the use of laboratory animals.

7 ACKNOWLEDGEMENTS

There are many people that I would like to acknowledge for their help and support throughout my Ph.D. studies and I would like to express my sincere gratitude to all of you.

First and foremost, I would like express my deepest gratitude to my main supervisor **Professor Magnus Ingelman-Sundberg**. I really appreciate all your support and encouragement over the years and for all the opportunities you have given me to work within so many interesting areas of research and collaborations. It has been both challenging and fun.

I also extend my sincere appreciation to my co-supervisors. It has been absolutely fantastic working with you! **Irene Edebert**, I can´t thank you enough for all your support, encouragement and enthusiasm throughout the years. Without you I would not have found my way into science. **Monica Ek**, thanks for your guidance in the beginning of my time in the lab. I was lucky to have you when everything was new and a bit confusing. **Etienne Neve**, your knowledge, experience and support has been invaluable to me. I am lucky to have you as my supervisor as well as my close friend.

A special thanks to **Ian Cotgreave** and his positive and supportive group at AstraZeneca in Gärtuna. Thanks for letting me be a part of your group and your interesting research.

I also wish to extend my appreciation to my collaborators:
The nice and talented people from Cellectis Stem Cells, Cellartis AB, in particular; **Petter Björquist**, **Janne Jensen**, **Jane Synnergren**, **Josefina Edsbagge** and **Gabriella Brolén**. To **Katrin Zeilinger's** group at Chartité University Hospital in Berlin, especially **Marc Lübberstedt** and **Thomas Schreiter**. Big thanks also to **Tommy B. Andersson**, **Malin Darnell** and **Maria Ulvestad** at AstraZeneca in Mölndal. It has been a real pleasure working with all of you and to learn from your expertise.

Big thanks to **Inger Johansson** for always taking the time to answer all my questions, both scientific and bureaucratic.

A very special thanks to my lab roomies; **Souren** and **Sussi**. Souren - for sharing your great knowledge and for just being such a gentleman. "Sushi" - for your help and for all the talks and laughs. Also, an extra special thanks to the both of you for putting up with me and Etienne and our many lively discussions.

A big hug to "the girls"; **Iren**, **Miyoung**, **Isa**, **Marina**, **Sandra**, **Sara** and **Ashley**. I have really enjoyed your company during parties, dinners and spa days. It has been so nice learning about your different cultures, especially the amazing food.

I would also like to express my appreciation to all the other nice colleagues in the MIS lab, both past and present, for creating such an enjoyable atmosphere in the lab. In particular, **Margareta, Anna**, **Sarah**, **Åsa**, **Maria**, **Ylva**, **Mike**, **Frank**, **Eva**, **Jia**, **Patrina**, **Sabrina**, **Isabel**, **Pedro**, **Pascal**, **Max**, **Claudia**, **Sara** and **Kristina**. Margareta - thank you for your great work and support. I have really enjoyed working with you. Anna - for being such a good friend. Sarah - for your strength and happy spirit. Åsa - for your energy and playfulness. The lab would not be the same without you girls. Frank – for all the fun and dancing. Maria – for being such a good friend and for serving champagne on a regular basis. Always fun with you and Erik! Ylva and Mike – for being so caring and easy going.

Big thanks to all the great **colleagues at FyFa** for being such a fun and supporting gang.

To the molecular biology team; **Linda**, **Martina** and **Maria**. That's where the science started. I also would like to acknowledge the **Toxicology class of 2006**.

I would also like to extend my appreciation to all my **friends outside of work**. A very special thanks goes out to my dear friend **Jeanette**, whom I consider myself very fortunate to have. Thank you for always being there for me. You know you can always count on me to do the same.

To the **Hagbard family** - for letting me be a part of your lovely little family, and to the **Johansson family** - for your incredible hospitality, and for your interest and enthusiasm in what I do. I'm glad I have you all in my life!

To my beloved family who is always there for me. My deepest gratitude goes to my parents who have given me all the support and every opportunity in life. My caring and strong **mother Helena**, who always encourages me to learn. My positive and social **father Anders**, who has taught me to enjoy life and to not always take things so seriously. To my beautiful sisters: **Petra**, **Ebba** and **Emelie**. I'm so glad we have each other. There's nothing like a sister when you want to talk, laugh or reflect on life. To all cherished extended family members, including those that are dearly missed. With a large family, you are definitely rich!

Finally, all my affection and gratitude to my beloved boys, **Niklas** and **Morgan**. I am so fortunate to share my life with you!

8 REFERENCES

1. Kola, I. and J. Landis, *Can the pharmaceutical industry reduce attrition rates?* Nat Rev Drug Discov, 2004. **3**(8): p. 711-5.
2. Moore, T.J., M.R. Cohen, and C.D. Furberg, *Serious adverse drug events reported to the Food and Drug Administration, 1998-2005.* Arch Intern Med, 2007. **167**(16): p. 1752-9.
3. Mjorndal, T., et al., *Adverse drug reactions as a cause for admissions to a department of internal medicine.* Pharmacoepidemiol Drug Saf, 2002. **11**(1): p. 65-72.
4. Wester, K., et al., *Incidence of fatal adverse drug reactions: a population based study.* Br J Clin Pharmacol, 2008. **65**(4): p. 573-9.
5. Schneeweiss, S., et al., *Admissions caused by adverse drug events to internal medicine and emergency departments in hospitals: a longitudinal population-based study.* Eur J Clin Pharmacol, 2002. **58**(4): p. 285-91.
6. Pirmohamed, M., et al., *Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients.* BMJ, 2004. **329**(7456): p. 15-9.
7. Lazarou, J., B.H. Pomeranz, and P.N. Corey, *Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies.* JAMA, 1998. **279**(15): p. 1200-5.
8. Eichelbaum, M., M. Ingelman-Sundberg, and W.E. Evans, *Pharmacogenomics and individualized drug therapy.* Annu Rev Med, 2006. **57**: p. 119-37.
9. Lasser, K.E., et al., *Timing of new black box warnings and withdrawals for prescription medications.* JAMA, 2002. **287**(17): p. 2215-20.
10. Kaplowitz, N., *Drug-induced liver disorders: implications for drug development and regulation.* Drug Saf, 2001. **24**(7): p. 483-90.
11. Pearson, H., *The bitterest pill.* Nature, 2006. **444**(7119): p. 532-3.
12. Need, A.C., A.G. Motulsky, and D.B. Goldstein, *Priorities and standards in pharmacogenetic research.* Nat Genet, 2005. **37**(7): p. 671-81.
13. Kaplowitz, N., *Idiosyncratic drug hepatotoxicity.* Nat Rev Drug Discov, 2005. **4**(6): p. 489-99.
14. Ballet, F., *Hepatotoxicity in drug development: detection, significance and solutions.* J Hepatol, 1997. **26 Suppl 2**: p. 26-36.
15. Olson, H., et al., *Concordance of the toxicity of pharmaceuticals in humans and in animals.* Regul Toxicol Pharmacol, 2000. **32**(1): p. 56-67.
16. Kubo, A., et al., *Development of definitive endoderm from embryonic stem cells in culture.* Development, 2004. **131**(7): p. 1651-62.
17. Kimelman, D. and K.J. Griffin, *Vertebrate mesendoderm induction and patterning.* Curr Opin Genet Dev, 2000. **10**(4): p. 350-6.
18. Shen, M.M., *Nodal signaling: developmental roles and regulation.* Development, 2007. **134**(6): p. 1023-34.
19. Nakanishi, M., et al., *Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium.* FASEB J, 2009. **23**(1): p. 114-22.
20. Zorn, A.M. and J.M. Wells, *Vertebrate endoderm development and organ formation.* Annu Rev Cell Dev Biol, 2009. **25**: p. 221-51.
21. Massague, J., J. Seoane, and D. Wotton, *Smad transcription factors.* Genes Dev, 2005. **19**(23): p. 2783-810.
22. Fei, T., et al., *Smad2 mediates Activin/Nodal signaling in mesendoderm differentiation of mouse embryonic stem cells.* Cell Res, 2010. **20**(12): p. 1306-18.
23. Kanai-Azuma, M., et al., *Depletion of definitive gut endoderm in Sox17-null mutant mice.* Development, 2002. **129**(10): p. 2367-2379.
24. Zorn, A.M. and J.M. Wells, *Molecular basis of vertebrate endoderm development.* Int Rev Cytol, 2007. **259**: p. 49-111.
25. Matsumoto, K., et al., *Liver organogenesis promoted by endothelial cells prior to vascular function.* Science, 2001. **294**(5542): p. 559-63.

26. Jung, J., et al., *Initiation of mammalian liver development from endoderm by fibroblast growth factors.* Science, 1999. **284**(5422): p. 1998-2003.
27. Rossi, J.M., et al., *Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm.* Genes Dev, 2001. **15**(15): p. 1998-2009.
28. Wauthier, E., et al., *Hepatic stem cells and hepatoblasts: identification, isolation, and ex vivo maintenance.* Methods Cell Biol, 2008. **86**: p. 137-225.
29. Lavon, N. and N. Benvenisty, *Study of hepatocyte differentiation using embryonic stem cells.* J Cell Biochem, 2005. **96**(6): p. 1193-202.
30. Zaret, K.S., *Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation.* Nat Rev Genet, 2008. **9**(5): p. 329-40.
31. Kamiya, A., T. Kinoshita, and A. Miyajima, *Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways.* FEBS Lett, 2001. **492**(1-2): p. 90-4.
32. Tanimizu, N. and A. Miyajima, *Molecular mechanism of liver development and regeneration.* Int Rev Cytol, 2007. **259**: p. 1-48.
33. Li, J., G. Ning, and S.A. Duncan, *Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha.* Genes Dev, 2000. **14**(4): p. 464-74.
34. Miller, M.S., et al., *Drug metabolic enzymes in developmental toxicology.* Fundam Appl Toxicol, 1996. **34**(2): p. 165-75.
35. *Stem Cells: Scientific Progress and Future Research Directions: National Institute of Health, In Stem Cell Information* 2001].
36. Dancygier, H., *Clinical Hepatology: Principles and Practice of Hepatobiliary Diseases*. Vol. Volume 1. 2010: Springer-Verlag Berlin Heidelberg.
37. Gebhardt, R., *Metabolic zonation of the liver: regulation and implications for liver function.* Pharmacol Ther, 1992. **53**(3): p. 275-354.
38. Gooding, P.E., et al., *Cytochrome P-450 distribution in rat liver and the effect of sodium phenobarbitone administration.* Chem Biol Interact, 1978. **20**(3): p. 299-310.
39. Bengtsson, G., et al., *Effect of phenobarbital on the distribution of drug metabolizing enzymes between periportal and perivenous rat hepatocytes prepared by digitonin-collagenase liver perfusion.* J Pharmacol Exp Ther, 1987. **240**(2): p. 663-7.
40. Li, Z. and A.M. Diehl, *Innate immunity in the liver.* Curr Opin Gastroenterol, 2003. **19**(6): p. 565-71.
41. Racanelli, V. and B. Rehermann, *The liver as an immunological organ.* Hepatology, 2006. **43**(2): p. S54-S62.
42. Lowes, K.N., et al., *Oval cell-mediated liver regeneration: Role of cytokines and growth factors.* J Gastroenterol Hepatol, 2003. **18**(1): p. 4-12.
43. Wanson, J.C., D. Bernaert, and C. May, *Morphology and functional properties of isolated and cultured hepatocytes.* Prog Liver Dis, 1979. **6**: p. 1-22.
44. Celton-Morizur, S., et al., *Polyploidy and liver proliferation: central role of insulin signaling.* Cell Cycle, 2010. **9**(3): p. 460-6.
45. Bertolino, P., M.C. Trescol-Biemont, and C. Rabourdin-Combe, *Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival.* Eur J Immunol, 1998. **28**(1): p. 221-36.
46. Overturf, K., et al., *The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion.* Am J Pathol, 1999. **155**(6): p. 2135-43.
47. Taub, R., *Liver regeneration: from myth to mechanism.* Nat Rev Mol Cell Biol, 2004. **5**(10): p. 836-47.
48. Thenappan, A., et al., *Role of transforming growth factor beta signaling and expansion of progenitor cells in regenerating liver.* Hepatology, 2010. **51**(4): p. 1373-82.
49. Friedman, S.L., *Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver.* Physiol Rev, 2008. **88**(1): p. 125-72.
50. Bilzer, M., F. Roggel, and A.L. Gerbes, *Role of Kupffer cells in host defense and liver disease.* Liver Int, 2006. **26**(10): p. 1175-86.

51. Muriel, P., *NF-kappaB in liver diseases: a target for drug therapy.* J Appl Toxicol, 2009. **29**(2): p. 91-100.
52. Fausto, N. and J.S. Campbell, *The role of hepatocytes and oval cells in liver regeneration and repopulation.* Mech Dev, 2003. **120**(1): p. 117-30.
53. Olynyk, J.K., et al., *Gadolinium chloride suppresses hepatic oval cell proliferation in rats with biliary obstruction.* Am J Pathol, 1998. **152**(2): p. 347-52.
54. Nagy, P., et al., *Dexamethasone inhibits the proliferation of hepatocytes and oval cells but not bile duct cells in rat liver.* Hepatology, 1998. **28**(2): p. 423-9.
55. Leite, A.R., et al., *Fibronectin and laminin induce expression of islet cell markers in hepatic oval cells in culture.* Cell Tissue Res, 2007. **327**(3): p. 529-37.
56. Kallis, Y.N., et al., *Remodelling of extracellular matrix is a requirement for the hepatic progenitor cell response.* Gut, 2011. **60**(4): p. 525-33.
57. Okaya, A., et al., *Oncostatin M inhibits proliferation of rat oval cells, OC15-5, inducing differentiation into hepatocytes.* Am J Pathol, 2005. **166**(3): p. 709-19.
58. Reynoso-Paz, S., et al., *The immunobiology of bile and biliary epithelium.* Hepatology, 1999. **30**(2): p. 351-7.
59. Padda, M.S., et al., *Drug-Induced Cholestasis.* Hepatology, 2011. **53**(4): p. 1377-1387.
60. Knolle, P.A. and A. Limmer, *Control of immune responses by scavenger liver endothelial cells.* Swiss Medical Weekly, 2003. **133**(37-38): p. 501-506.
61. Limmer, A., et al., *Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance.* European Journal of Immunology, 2005. **35**(10): p. 2970-2981.
62. Wake, K., *"Sternzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A.* Am J Anat, 1971. **132**(4): p. 429-62.
63. Gutierrez-Ruiz, M.C. and L.E. Gomez-Quiroz, *Liver fibrosis: searching for cell model answers.* Liver Int, 2007. **27**(4): p. 434-9.
64. Paik, Y.H., et al., *Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells.* Hepatology, 2003. **37**(5): p. 1043-55.
65. Vinas, O., et al., *Human hepatic stellate cells show features of antigen-presenting cells and stimulate lymphocyte proliferation.* Hepatology, 2003. **38**(4): p. 919-29.
66. Unanue, E.R., *Ito cells, stellate cells, and myofibroblasts: new actors in antigen presentation.* Immunity, 2007. **26**(1): p. 9-10.
67. Winau, F., et al., *Ito cells are liver-resident antigen-presenting cells for activating T cell responses.* Immunity, 2007. **26**(1): p. 117-29.
68. Senoo, H., N. Kojima, and M. Sato, *Vitamin A-storing cells (stellate cells).* Vitam Horm, 2007. **75**: p. 131-59.
69. Mehal, W.Z., F. Azzaroli, and I.N. Crispe, *Immunology of the healthy liver: old questions and new insights.* Gastroenterology, 2001. **120**(1): p. 250-60.
70. Wake, K., et al., *Cell biology and kinetics of Kupffer cells in the liver.* Int Rev Cytol, 1989. **118**: p. 173-229.
71. Gale, R.P., R.S. Sparkes, and D.W. Golde, *Bone marrow origin of hepatic macrophages (Kupffer cells) in humans.* Science, 1978. **201**(4359): p. 937-8.
72. Naito, M., et al., *Differentiation and function of Kupffer cells.* Med Electron Microsc, 2004. **37**(1): p. 16-28.
73. Gregory, S.H. and E.J. Wing, *Neutrophil-Kupffer cell interaction: a critical component of host defenses to systemic bacterial infections.* J Leukoc Biol, 2002. **72**(2): p. 239-48.
74. Thornton, A.J., J. Ham, and S.L. Kunkel, *Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte cultures.* Hepatology, 1991. **14**(6): p. 1112-22.
75. Kimura, M., et al., *Female predominance of extrathymic T cells in mice: statistical analysis.* Immunol Lett, 1994. **39**(3): p. 259-67.
76. Tsukahara, A., et al., *Mouse liver T cells: their change with aging and in comparison with peripheral T cells.* Hepatology, 1997. **26**(2): p. 301-9.

77. Vermijlen, D., et al., *Pit cells (Hepatic natural killer cells) of the rat induce apoptosis in colon carcinoma cells by the perforin/granzyme pathway.* Hepatology, 1999. **29**(1): p. 51-6.
78. Doherty, D.G., et al., *The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns.* J Immunol, 1999. **163**(4): p. 2314-21.
79. Welsh, R.M., et al., *Alpha beta and gamma delta T-cell networks and their roles in natural resistance to viral infections.* Immunol Rev, 1997. **159**: p. 79-93.
80. Geissmann, F., et al., *Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids.* PLoS Biol, 2005. **3**(4): p. e113.
81. Gutcher, I. and B. Becher, *APC-derived cytokines and T cell polarization in autoimmune inflammation.* J Clin Invest, 2007. **117**(5): p. 1119-27.
82. Holt, M.P. and C. Ju, *Mechanisms of drug-induced liver injury.* Aaps Journal, 2006. **8**(1): p. E48-E54.
83. Knolle, P.A., et al., *Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells.* Gastroenterology, 1999. **116**(6): p. 1428-40.
84. Limmer, A., et al., *Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance.* Nat Med, 2000. **6**(12): p. 1348-54.
85. Xu, C., C.Y. Li, and A.N. Kong, *Induction of phase I, II and III drug metabolism/transport by xenobiotics.* Arch Pharm Res, 2005. **28**(3): p. 249-68.
86. Liska, D.J., *The detoxification enzyme systems.* Altern Med Rev, 1998. **3**(3): p. 187-98.
87. Nebert, D.W. and T.P. Dalton, *The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis.* Nat Rev Cancer, 2006. **6**(12): p. 947-60.
88. Park, B.K., M. Pirmohamed, and N.R. Kitteringham, *The role of cytochrome P450 enzymes in hepatic and extrahepatic human drug toxicity.* Pharmacol Ther, 1995. **68**(3): p. 385-424.
89. Castell, J.V., et al., *Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies.* Expert Opin Drug Metab Toxicol, 2006. **2**(2): p. 183-212.
90. Klingenberg, M., *Pigments of rat liver microsomes.* Arch Biochem Biophys, 1958. **75**(2): p. 376-86.
91. Garfinkel, D., *Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions.* Arch Biochem Biophys, 1958. **77**(2): p. 493-509.
92. Omura, T. and R. Sato, *A new cytochrome in liver microsomes.* J Biol Chem, 1962. **237**: p. 1375-6.
93. Guengerich, F.P., *Characterization of human cytochrome P450 enzymes.* FASEB J, 1992. **6**(2): p. 745-8.
94. Werck-Reichhart, D. and R. Feyereisen, *Cytochromes P450: a success story.* Genome Biol, 2000. **1**(6): p. REVIEWS3003.
95. Porter, T.D., *The roles of cytochrome b5 in cytochrome P450 reactions.* J Biochem Mol Toxicol, 2002. **16**(6): p. 311-6.
96. Gan, L., et al., *Role of NADPH-cytochrome P450 reductase and cytochrome-b5/NADH-b5 reductase in variability of CYP3A activity in human liver microsomes.* Drug Metab Dispos, 2009. **37**(1): p. 90-6.
97. Nebert, D.W., et al., *The P450 gene superfamily: recommended nomenclature.* DNA, 1987. **6**(1): p. 1-11.
98. Michalets, E.L., *Update: clinically significant cytochrome P-450 drug interactions.* Pharmacotherapy, 1998. **18**(1): p. 84-112.
99. Guengerich, F.P., *Cytochrome p450 and chemical toxicology.* Chem Res Toxicol, 2008. **21**(1): p. 70-83.
100. Johansson, I. and M. Ingelman-Sundberg, *Genetic polymorphism and toxicology--with emphasis on cytochrome p450.* Toxicol Sci, 2011. **120**(1): p. 1-13.
101. Evans, W.E. and M.V. Relling, *Pharmacogenomics: translating functional genomics into rational therapeutics.* Science, 1999. **286**(5439): p. 487-91.

102. Ingelman-Sundberg, M., et al., *Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects.* Pharmacol Ther, 2007. **116**(3): p. 496-526.
103. Ingelman-Sundberg, M., *Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity.* Pharmacogenomics J, 2005. **5**(1): p. 6-13.
104. Sotaniemi, E.A., et al., *Age and cytochrome P450-linked drug metabolism in humans: an analysis of 226 subjects with equal histopathologic conditions.* Clin Pharmacol Ther, 1997. **61**(3): p. 331-9.
105. Wolbold, R., et al., *Sex is a major determinant of CYP3A4 expression in human liver.* Hepatology, 2003. **38**(4): p. 978-88.
106. Martinez-Jimenez, C.P., et al., *Transcriptional regulation and expression of CYP3A4 in hepatocytes.* Curr Drug Metab, 2007. **8**(2): p. 185-94.
107. Lamba, V., et al., *PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators.* Toxicol Appl Pharmacol, 2004. **199**(3): p. 251-65.
108. Rodriguez-Antona, C., et al., *Molecular genetics and epigenetics of the cytochrome P450 gene family and its relevance for cancer risk and treatment.* Hum Genet, 2010. **127**(1): p. 1-17.
109. Shimada, T., et al., *Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians.* J Pharmacol Exp Ther, 1994. **270**(1): p. 414-23.
110. Eichelbaum, M. and O. Burk, *CYP3A genetics in drug metabolism.* Nat Med, 2001. **7**(3): p. 285-7.
111. Guengerich, F.P., *Cytochrome P-450 3A4: regulation and role in drug metabolism.* Annu Rev Pharmacol Toxicol, 1999. **39**: p. 1-17.
112. Kawano, S., et al., *Purification of human liver cytochrome P-450 catalyzing testosterone 6 beta-hydroxylation.* J Biochem, 1987. **102**(3): p. 493-501.
113. Niwa, T., et al., *Contribution of human hepatic cytochrome P450 isoforms to regioselective hydroxylation of steroid hormones.* Xenobiotica, 1998. **28**(6): p. 539-47.
114. Bodin, K., et al., *Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4.* J Biol Chem, 2001. **276**(42): p. 38685-9.
115. Westlind, A., et al., *Interindividual differences in hepatic expression of CYP3A4: relationship to genetic polymorphism in the 5'-upstream regulatory region.* Biochem Biophys Res Commun, 1999. **259**(1): p. 201-5.
116. Rodriguez-Antona, C., et al., *Phenotype-genotype variability in the human CYP3A locus as assessed by the probe drug quinine and analyses of variant CYP3A4 alleles.* Biochem Biophys Res Commun, 2005. **338**(1): p. 299-305.
117. Felix, C.A., et al., *Association of CYP3A4 genotype with treatment-related leukemia.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13176-81.
118. Floyd, M.D., et al., *Genotype-phenotype associations for common CYP3A4 and CYP3A5 variants in the basal and induced metabolism of midazolam in European- and African-American men and women.* Pharmacogenetics, 2003. **13**(10): p. 595-606.
119. Moore, L.B., et al., *St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor.* Proc Natl Acad Sci U S A, 2000. **97**(13): p. 7500-2.
120. Burk, O. and L. Wojnowski, *Cytochrome P450 3A and their regulation.* Naunyn Schmiedebergs Arch Pharmacol, 2004. **369**(1): p. 105-24.
121. Finta, C. and P.G. Zaphiropoulos, *The human cytochrome P450 3A locus. Gene evolution by capture of downstream exons.* Gene, 2000. **260**(1-2): p. 13-23.
122. Goodwin, B., E. Hodgson, and C. Liddle, *The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module.* Mol Pharmacol, 1999. **56**(6): p. 1329-39.

123. Zhang, J., et al., *The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants.* Pharmacogenetics, 2001. **11**(7): p. 555-72.
124. Matsumura, K., et al., *Identification of a novel polymorphic enhancer of the human CYP3A4 gene.* Mol Pharmacol, 2004. **65**(2): p. 326-34.
125. Wang, Y.M., et al., *Role of CAR and PXR in xenobiotic sensing and metabolism.* Expert Opin Drug Metab Toxicol, 2012. **8**(7): p. 803-17.
126. Bertilsson, G., et al., *Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction.* Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12208-13.
127. Kliewer, S.A., et al., *An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway.* Cell, 1998. **92**(1): p. 73-82.
128. Lehmann, J.M., et al., *The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions.* J Clin Invest, 1998. **102**(5): p. 1016-23.
129. Goodwin, B., et al., *Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor.* Mol Pharmacol, 2002. **62**(2): p. 359-65.
130. Drocourt, L., et al., *Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes.* J Biol Chem, 2002. **277**(28): p. 25125-32.
131. Pascussi, J.M., et al., *Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor.* Eur J Biochem, 2001. **268**(24): p. 6346-58.
132. Tirona, R.G., et al., *The orphan nuclear receptor HNF4 alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4.* Nature Medicine, 2003. **9**(2): p. 220-224.
133. Rodriguez-Antona, C., et al., *Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein alpha and hepatocyte nuclear factor-3 gamma.* Mol Pharmacol, 2003. **63**(5): p. 1180-9.
134. Kacevska, M., et al., *DNA methylation dynamics in the hepatic CYP3A4 gene promoter.* Biochimie, 2012.
135. Li, Y., et al., *Dynamic patterns of histone methylation are associated with ontogenic expression of the Cyp3a genes during mouse liver maturation.* Mol Pharmacol, 2009. **75**(5): p. 1171-9.
136. Pan, Y.Z., W. Gao, and A.M. Yu, *MicroRNAs regulate CYP3A4 expression via direct and indirect targeting.* Drug Metab Dispos, 2009. **37**(10): p. 2112-7.
137. Takagi, S., et al., *Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4.* J Biol Chem, 2008. **283**(15): p. 9674-80.
138. Lichti-Kaiser, K., C. Xu, and J.L. Staudinger, *Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner.* J Biol Chem, 2009. **284**(11): p. 6639-49.
139. Ding, X. and J.L. Staudinger, *Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C.* Biochem Pharmacol, 2005. **69**(5): p. 867-73.
140. Wang, Y., et al., *A role for protein phosphorylation in cytochrome P450 3A4 ubiquitin-dependent proteasomal degradation.* J Biol Chem, 2009. **284**(9): p. 5671-84.
141. Ma, X., J.R. Idle, and F.J. Gonzalez, *The pregnane X receptor: from bench to bedside.* Expert Opin Drug Metab Toxicol, 2008. **4**(7): p. 895-908.
142. Staudinger, J., et al., *Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor.* Drug Metab Dispos, 2001. **29**(11): p. 1467-72.
143. Tirona, R.G. and R.B. Kim, *Nuclear receptors and drug disposition gene regulation.* J Pharm Sci, 2005. **94**(6): p. 1169-86.
144. Kojima, K., et al., *Broad but distinct role of pregnane x receptor on the expression of individual cytochrome p450s in human hepatocytes.* Drug Metab Pharmacokinet, 2007. **22**(4): p. 276-86.

145. Goodwin, B., et al., *Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor.* Mol Pharmacol, 2001. **60**(3): p. 427-31.
146. Gardner-Stephen, D., et al., *Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression.* Drug Metab Dispos, 2004. **32**(3): p. 340-7.
147. Geick, A., M. Eichelbaum, and O. Burk, *Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin.* J Biol Chem, 2001. **276**(18): p. 14581-7.
148. Kast, H.R., et al., *Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor.* J Biol Chem, 2002. **277**(4): p. 2908-15.
149. Gu, X., et al., *Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents.* J Biol Chem, 2006. **281**(26): p. 17882-9.
150. Koyano, S., et al., *Functional characterization of four naturally occurring variants of human pregnane X receptor (PXR): one variant causes dramatic loss of both DNA binding activity and the transactivation of the CYP3A4 promoter/enhancer region.* Drug Metab Dispos, 2004. **32**(1): p. 149-54.
151. Squires, E.J., T. Sueyoshi, and M. Negishi, *Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver.* J Biol Chem, 2004. **279**(47): p. 49307-14.
152. Kawana, K., et al., *Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR.* Mol Pharmacol, 2003. **63**(3): p. 524-31.
153. Liu, F.J., et al., *The far and distal enhancers in the CYP3A4 gene co-ordinate the proximal promoter in responding similarly to the pregnane X receptor but differentially to hepatocyte nuclear factor-4alpha.* Biochem J, 2008. **409**(1): p. 243-50.
154. Toriyabe, T., et al., *Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics.* Molecular Pharmacology, 2009. **75**(3): p. 677-84.
155. Biswas, A., et al., *Acetylation of pregnane X receptor protein determines selective function independent of ligand activation.* Biochem Biophys Res Commun, 2011. **406**(3): p. 371-6.
156. Staudinger, J.L., et al., *Post-translational modification of pregnane x receptor.* Pharmacol Res, 2011. **64**(1): p. 4-10.
157. Istrate, M.A., et al., *Regulation of CYP3A4 by pregnane X receptor: The role of nuclear receptors competing for response element binding.* Biochem Biophys Res Commun, 2010. **393**(4): p. 688-93.
158. Li, T. and J.Y. Chiang, *Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression.* Drug Metab Dispos, 2006. **34**(5): p. 756-64.
159. Masuyama, H., et al., *Ligands have various potential effects on the degradation of pregnane X receptor by proteasome.* Endocrinology, 2002. **143**(1): p. 55-61.
160. Ding, X. and J.L. Staudinger, *Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway.* J Pharmacol Exp Ther, 2005. **312**(2): p. 849-56.
161. Xie, Y., et al., *Epigenetic regulation of transcriptional activity of pregnane X receptor by protein arginine methyltransferase 1.* J Biol Chem, 2009. **284**(14): p. 9199-205.
162. Hustert, E., et al., *Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4.* Drug Metab Dispos, 2001. **29**(11): p. 1454-9.
163. Lamba, V., et al., *Genetic predictors of interindividual variability in hepatic CYP3A4 expression.* J Pharmacol Exp Ther, 2010. **332**(3): p. 1088-99.
164. Fukuen, S., et al., *Identification of the novel splicing variants for the hPXR in human livers.* Biochem Biophys Res Commun, 2002. **298**(3): p. 433-8.

165. Lin, Y.S., et al., *The major human pregnane X receptor (PXR) splice variant, PXR.2, exhibits significantly diminished ligand-activated transcriptional regulation.* Drug Metab Dispos, 2009. **37**(6): p. 1295-304.
166. Gomez-Lechon, M.J., J.V. Castell, and M.T. Donato, *The use of hepatocytes to investigate drug toxicity.* Methods Mol Biol, 2010. **640**: p. 389-415.
167. Chen, Y., et al., *Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture.* Hepatology, 2012. **55**(2): p. 563-74.
168. Donato, M.T., et al., *Cell lines: a tool for in vitro drug metabolism studies.* Curr Drug Metab, 2008. **9**(1): p. 1-11.
169. Bader, A., et al., *A stable long-term hepatocyte culture system for studies of physiologic processes: cytokine stimulation of the acute phase response in rat and human hepatocytes.* Biotechnol Prog, 1992. **8**(3): p. 219-25.
170. LeCluyse, E.L., et al., *Cultured rat hepatocytes.* Pharm Biotechnol, 1996. **8**: p. 121-59.
171. Li, M., et al., *Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human.* Eur J Pharm Sci, 2008. **35**(1-2): p. 114-26.
172. Wilkening, S., F. Stahl, and A. Bader, *Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties.* Drug Metab Dispos, 2003. **31**(8): p. 1035-42.
173. Goldring, C.E., et al., *Development of a transactivator in hepatoma cells that allows expression of phase I, phase II, and chemical defense genes.* Am J Physiol Cell Physiol, 2006. **290**(1): p. C104-15.
174. Parent, R., et al., *Origin and characterization of a human bipotent liver progenitor cell line.* Gastroenterology, 2004. **126**(4): p. 1147-56.
175. Aninat, C., et al., *Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells.* Drug Metab Dispos, 2006. **34**(1): p. 75-83.
176. Kanebratt, K.P. and T.B. Andersson, *Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies.* Drug Metab Dispos, 2008. **36**(7): p. 1444-52.
177. Fuchs, E., T. Tumbar, and G. Guasch, *Socializing with the neighbors: stem cells and their niche.* Cell, 2004. **116**(6): p. 769-78.
178. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
179. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.
180. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
181. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
182. Pesce, M. and H.R. Scholer, *Oct-4: gatekeeper in the beginnings of mammalian development.* Stem Cells, 2001. **19**(4): p. 271-8.
183. Avilion, A.A., et al., *Multipotent cell lineages in early mouse development depend on SOX2 function.* Genes Dev, 2003. **17**(1): p. 126-40.
184. Chambers, I., et al., *Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells.* Cell, 2003. **113**(5): p. 643-55.
185. Pera, M.F. and A.O. Trounson, *Human embryonic stem cells: prospects for development.* Development, 2004. **131**(22): p. 5515-25.
186. Jensen, J., J. Hyllner, and P. Bjorquist, *Human embryonic stem cell technologies and drug discovery.* J Cell Physiol, 2009. **219**(3): p. 513-9.
187. Yildirimman, R., et al., *Human embryonic stem cell derived hepatocyte-like cells as a tool for in vitro hazard assessment of chemical carcinogenicity.* Toxicol Sci, 2011. **124**(2): p. 278-90.

188. Greenhough, S., C.N. Medine, and D.C. Hay, *Pluripotent stem cell derived hepatocyte like cells and their potential in toxicity screening.* Toxicology, 2010. **278**(3): p. 250-5.
189. D'Amour, K.A., et al., *Efficient differentiation of human embryonic stem cells to definitive endoderm.* Nat Biotechnol, 2005. **23**(12): p. 1534-41.
190. Hay, D.C., et al., *Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling.* Proc Natl Acad Sci U S A, 2008. **105**(34): p. 12301-6.
191. Touboul, T., et al., *Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development.* Hepatology, 2010. **51**(5): p. 1754-65.
192. Cai, J., et al., *Directed differentiation of human embryonic stem cells into functional hepatic cells.* Hepatology, 2007. **45**(5): p. 1229-39.
193. Duan, Y., et al., *Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells.* Stem Cells, 2010. **28**(4): p. 674-86.
194. Wilson, J.W. and E.H. Leduc, *Role of cholangioles in restoration of the liver of the mouse after dietary injury.* J Pathol Bacteriol, 1958. **76**(2): p. 441-9.
195. Herrera, M.B., et al., *Isolation and characterization of a stem cell population from adult human liver.* Stem Cells, 2006. **24**(12): p. 2840-50.
196. Chen, Q.R., et al., *[In-vitro amplification of oval cells with preservation of stem cell phenotype].* Zhonghua Bing Li Xue Za Zhi, 2010. **39**(8): p. 548-52.
197. Carraro, A., et al., *A combining method to enhance the in vitro differentiation of hepatic precursor cells.* Tissue Eng Part C Methods, 2010. **16**(6): p. 1543-51.
198. Zhou, W.L., et al., *Stem cell differentiation and human liver disease.* World J Gastroenterol, 2012. **18**(17): p. 2018-25.
199. Yamanaka, S., *A Fresh Look at iPS Cells.* Cell, 2009. **137**(1): p. 13-17.
200. Si-Tayeb, K., et al., *Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells.* Hepatology, 2010. **51**(1): p. 297-305.
201. Sullivan, G.J., et al., *Generation of functional human hepatic endoderm from human induced pluripotent stem cells.* Hepatology, 2010. **51**(1): p. 329-35.
202. Takayama, K., et al., *Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4alpha transduction.* Mol Ther, 2012. **20**(1): p. 127-37.
203. Lee, S.B., et al., *Contribution of hepatic lineage stage-specific donor memory to the differential potential of induced mouse pluripotent stem cells.* Stem Cells, 2012. **30**(5): p. 997-1007.
204. Dambach, D.M., B.A. Andrews, and F. Moulin, *New technologies and screening strategies for hepatotoxicity: use of in vitro models.* Toxicol Pathol, 2005. **33**(1): p. 17-26.
205. Bissell, D.M., et al., *Drug-induced liver injury: mechanisms and test systems.* Hepatology, 2001. **33**(4): p. 1009-13.
206. Lee, W.M., *Drug-induced hepatotoxicity.* N Engl J Med, 2003. **349**(5): p. 474-85.
207. Roth, R.A. and P.E. Ganey, *Intrinsic versus idiosyncratic drug-induced hepatotoxicity--two villains or one?* J Pharmacol Exp Ther, 2010. **332**(3): p. 692-7.
208. Lee, K.S., et al., *Assessment of reactive metabolites in drug-induced liver injury.* Arch Pharm Res, 2011. **34**(11): p. 1879-86.
209. Simpson, K.J., et al., *Cytokines and the liver.* J Hepatol, 1997. **27**(6): p. 1120-32.
210. Naisbitt, D.J., et al., *Reactive metabolites and their role in drug reactions.* Curr Opin Allergy Clin Immunol, 2001. **1**(4): p. 317-25.
211. Alfirevic, A. and M. Pirmohamed, *Drug Induced Hypersensitivity and the HLA Complex.* pharmaceuticals, 2011. **4**: p. 69-90.
212. Ikeda, T., *Drug-induced idiosyncratic hepatotoxicity: prevention strategy developed after the troglitazone case.* Drug Metab Pharmacokinet, 2011. **26**(1): p. 60-70.
213. Daly, A.K., et al., *HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin.* Nat Genet, 2009. **41**(7): p. 816-9.

214. Kindmark, A., et al., *Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis.* Pharmacogenomics J, 2008. **8**(3): p. 186-95.
215. Lucena, M.I., et al., *Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles.* Gastroenterology, 2011. **141**(1): p. 338-47.
216. Singer, J.B., et al., *A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury.* Nat Genet, 2010. **42**(8): p. 711-4.
217. Gomez-Lechon, M.J., J.V. Castell, and M.T. Donato, *An update on metabolism studies using human hepatocytes in primary culture.* Expert Opin Drug Metab Toxicol, 2008. **4**(7): p. 837-54.
218. Machide, M., et al., *Contact inhibition of hepatocyte growth regulated by functional association of the c-Met/hepatocyte growth factor receptor and LAR protein-tyrosine phosphatase.* J Biol Chem, 2006. **281**(13): p. 8765-72.
219. Dvir-Ginzberg, M., et al., *Liver tissue engineering within alginate scaffolds: effects of cell-seeding density on hepatocyte viability, morphology, and function.* Tissue Eng, 2003. **9**(4): p. 757-66.
220. Vickers, A.E., et al., *Cyclosporin A metabolism in human liver, kidney, and intestine slices. Comparison to rat and dog slices and human cell lines.* Drug Metab Dispos, 1992. **20**(6): p. 802-9.
221. Schumacher, K., et al., *Perfusion culture improves the maintenance of cultured liver tissue slices.* Tissue Eng, 2007. **13**(1): p. 197-205.
222. Park, T.G., *Perfusion culture of hepatocytes within galactose-derivatized biodegradable poly(lactide-co-glycolide) scaffolds prepared by gas foaming of effervescent salts.* J Biomed Mater Res, 2002. **59**(1): p. 127-35.
223. Domansky, K., et al., *Perfused multiwell plate for 3D liver tissue engineering.* Lab Chip, 2010. **10**(1): p. 51-8.
224. Tostoes, R.M., et al., *Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing.* Hepatology, 2012. **55**(4): p. 1227-36.
225. Powers, M.J., et al., *Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor.* Tissue Eng, 2002. **8**(3): p. 499-513.
226. De Bartolo, L., et al., *Human hepatocyte functions in a crossed hollow fiber membrane bioreactor.* Biomaterials, 2009. **30**(13): p. 2531-43.
227. Schmelzer, E., et al., *Effect of human patient plasma ex vivo treatment on gene expression and progenitor cell activation of primary human liver cells in multi-compartment 3D perfusion bioreactors for extra-corporeal liver support.* Biotechnol Bioeng, 2009. **103**(4): p. 817-27.
228. Zeilinger, K., et al., *Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies.* Tissue Eng Part C Methods, 2011. **17**(5): p. 549-56.
229. Donato, M.T., J.V. Castell, and M.J. Gomez-Lechon, *Co-cultures of hepatocytes with epithelial-like cell lines: expression of drug-biotransformation activities by hepatocytes.* Cell Biol Toxicol, 1991. **7**(1): p. 1-14.
230. Bhandari, R.N., et al., *Liver tissue engineering: a role for co-culture systems in modifying hepatocyte function and viability.* Tissue Eng, 2001. **7**(3): p. 345-57.
231. Riccalton-Banks, L., et al., *Long-term culture of functional liver tissue: three-dimensional coculture of primary hepatocytes and stellate cells.* Tissue Eng, 2003. **9**(3): p. 401-10.
232. Nieto, N., S.L. Friedman, and A.I. Cederbaum, *Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species.* Hepatology, 2002. **35**(1): p. 62-73.
233. Tukov, F.F., et al., *Modeling inflammation-drug interactions in vitro: a rat Kupffer cell-hepatocyte coculture system.* Toxicol In Vitro, 2006. **20**(8): p. 1488-99.
234. Alabraba, E.B., et al., *Coculture of human liver macrophages and cholangiocytes leads to CD40-dependent apoptosis and cytokine secretion.* Hepatology, 2008. **47**(2): p. 552-62.

235. Aden, D.P., et al., *Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line.* Nature, 1979. **282**(5739): p. 615-6.
236. Nakabayashi, H., et al., *Growth of human hepatoma cells lines with differentiated functions in chemically defined medium.* Cancer Res, 1982. **42**(9): p. 3858-63.
237. Tsuchiya, S., et al., *Establishment and characterization of a human acute monocytic leukemia cell line (THP-1).* Int J Cancer, 1980. **26**(2): p. 171-6.
238. Heins, N., et al., *Derivation, characterization, and differentiation of human embryonic stem cells.* Stem Cells, 2004. **22**(3): p. 367-76.
239. Heins, N., et al., *Clonal derivation and characterization of human embryonic stem cell lines.* J Biotechnol, 2006. **122**(4): p. 511-20.
240. Michael, S.L., et al., *Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species.* Hepatology, 1999. **30**(1): p. 186-95.
241. Muriel, P. and Y. Escobar, *Kupffer cells are responsible for liver cirrhosis induced by carbon tetrachloride.* J Appl Toxicol, 2003. **23**(2): p. 103-8.
242. Rachek, L.I., et al., *Troglitazone, but not rosiglitazone, damages mitochondrial DNA and induces mitochondrial dysfunction and cell death in human hepatocytes.* Toxicol Appl Pharmacol, 2009. **240**(3): p. 348-54.
243. Gerlach, J.C., et al., *Bioreactor for a larger scale hepatocyte in vitro perfusion.* Transplantation, 1994. **58**(9): p. 984-8.
244. Sauer, I.M., et al., *Extracorporeal liver support based on primary human liver cells and albumin dialysis--treatment of a patient with primary graft non-function.* J Hepatol, 2003. **39**(4): p. 649-53.
245. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
246. Pease, A.C., et al., *Light-generated oligonucleotide arrays for rapid DNA sequence analysis.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 5022-6.
247. Pradervand, S., et al., *Affymetrix Whole-Transcript Human Gene 1.0 ST array is highly concordant with standard 3' expression arrays.* Biotechniques, 2008. **44**(6): p. 759-62.
248. Dennis, G., Jr., et al., *DAVID: Database for Annotation, Visualization, and Integrated Discovery.* Genome Biol, 2003. **4**(5): p. P3.
249. Huang da, W., et al., *DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists.* Nucleic Acids Res, 2007. **35**(Web Server issue): p. W169-75.
250. Wang, Y., et al., *Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays.* BMC Genomics, 2006. **7**: p. 59.
251. Claude, A., *The Constitution of Protoplasm.* Science, 1943. **97**(2525): p. 451-6.
252. Tegude, H., et al., *Molecular mechanism of basal CYP3A4 regulation by hepatocyte nuclear factor 4alpha: evidence for direct regulation in the intestine.* Drug Metab Dispos, 2007. **35**(6): p. 946-54.
253. Burnette, W.N., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.* Anal Biochem, 1981. **112**(2): p. 195-203.
254. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
255. Gerets, H.H., et al., *Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins.* Cell Biol Toxicol, 2012. **28**(2): p. 69-87.
256. Tilg, H. and A.M. Diehl, *Cytokines in alcoholic and nonalcoholic steatohepatitis.* N Engl J Med, 2000. **343**(20): p. 1467-76.
257. Thurman, R.G., *II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin.* Am J Physiol, 1998. **275**(4 Pt 1): p. G605-11.
258. Billiar, T.R., et al., *Kupffer cell:hepatocyte cocultures release nitric oxide in response to bacterial endotoxin.* J Surg Res, 1990. **48**(4): p. 349-53.

259. Steinhorn, D.M. and F.B. Cerra, *Comparative effects of lipopolysaccharide on newborn versus adult rat hepatocyte and nonparenchymal cell cocultures.* Crit Care Med, 1997. **25**(1): p. 121-7.
260. Guo, L., et al., *Differences in hepatotoxicity and gene expression profiles by anti-diabetic PPAR gamma agonists on rat primary hepatocytes and human HepG2 cells.* Mol Divers, 2006. **10**(3): p. 349-60.
261. Smith, M.T., *Mechanisms of troglitazone hepatotoxicity.* Chem Res Toxicol, 2003. **16**(6): p. 679-87.
262. Masubuchi, Y., *Metabolic and non-metabolic factors determining troglitazone hepatotoxicity: a review.* Drug Metab Pharmacokinet, 2006. **21**(5): p. 347-56.
263. Carriere, A., et al., *Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect.* J Biol Chem, 2004. **279**(39): p. 40462-9.
264. Vansant, G., et al., *Gene expression analysis of troglitazone reveals its impact on multiple pathways in cell culture: a case for in vitro platforms combined with gene expression analysis for early (idiosyncratic) toxicity screening.* Int J Toxicol, 2006. **25**(2): p. 85-94.
265. Reinecke, F., et al., *Metallothionein isoform 2A expression is inducible and protects against ROS-mediated cell death in rotenone-treated HeLa cells.* Biochem J, 2006. **395**(2): p. 405-15.
266. Arya, R., M. Mallik, and S.C. Lakhotia, *Heat shock genes - integrating cell survival and death.* J Biosci, 2007. **32**(3): p. 595-610.
267. Bai, J. and A.I. Cederbaum, *Mitochondrial catalase and oxidative injury.* Biol Signals Recept, 2001. **10**(3-4): p. 189-99.
268. Ramachandran, V., et al., *Troglitazone increases cytochrome P-450 3A protein and activity in primary cultures of human hepatocytes.* Drug Metab Dispos, 1999. **27**(10): p. 1194-9.
269. Haskins, J.R., et al., *Thiazolidinedione toxicity to isolated hepatocytes revealed by coherent multiprobe fluorescence microscopy and correlated with multiparameter flow cytometry of peripheral leukocytes.* Arch Toxicol, 2001. **75**(7): p. 425-38.
270. Bova, M.P., et al., *Troglitazone induces a rapid drop of mitochondrial membrane potential in liver HepG2 cells.* Toxicol Lett, 2005. **155**(1): p. 41-50.
271. Roth, R.A., et al., *Inflammation and drug idiosyncrasy--is there a connection?* J Pharmacol Exp Ther, 2003. **307**(1): p. 1-8.
272. Luster, A.D. and P. Leder, *IP-10, a -C-X-C- chemokine, elicits a potent thymus-dependent antitumor response in vivo.* J Exp Med, 1993. **178**(3): p. 1057-65.
273. Geiser, T., et al., *The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes.* J Biol Chem, 1993. **268**(21): p. 15419-24.
274. Blazka, M.E., et al., *Role of proinflammatory cytokines in acetaminophen hepatotoxicity.* Toxicol Appl Pharmacol, 1995. **133**(1): p. 43-52.
275. Schwabe, R.F. and D.A. Brenner, *Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(4): p. G583-9.
276. Jiang, C., A.T. Ting, and B. Seed, *PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines.* Nature, 1998. **391**(6662): p. 82-6.
277. Ricote, M., et al., *The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation.* Nature, 1998. **391**(6662): p. 79-82.
278. Wang, Y., et al., *Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates.* Hepatology, 2010. **52**(4): p. 1443-54.
279. Yamashina, S., et al., *Ethanol-induced sensitization to endotoxin in Kupffer cells is dependent upon oxidative stress.* Alcohol Clin Exp Res, 2005. **29**(12 Suppl): p. 246S-50S.
280. Buchweitz, J.P., et al., *Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy?* J Pharmacol Exp Ther, 2002. **300**(2): p. 460-7.

281. Hansen, J., D.L. Cherwitz, and J.I. Allen, *The role of tumor necrosis factor-alpha in acute endotoxin-induced hepatotoxicity in ethanol-fed rats.* Hepatology, 1994. **20**(2): p. 461-74.
282. Rambhatla, L., et al., *Generation of hepatocyte-like cells from human embryonic stem cells.* Cell Transplant, 2003. **12**(1): p. 1-11.
283. Chen, Y.F., et al., *Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol.* Hepatology, 2012. **55**(4): p. 1193-203.
284. Takayama, K., et al., *Efficient and directive generation of two distinct endoderm lineages from human ESCs and iPSCs by differentiation stage-specific SOX17 transduction.* PLoS One, 2011. **6**(7): p. e21780.
285. Zernicka-Goetz, M., *Patterning of the embryo: the first spatial decisions in the life of a mouse.* Development, 2002. **129**(4): p. 815-29.
286. Yasunaga, M., et al., *Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells.* Nat Biotechnol, 2005. **23**(12): p. 1542-50.
287. Synnergren, J., et al., *Transcriptional profiling of human embryonic stem cells differentiating to definitive and primitive endoderm and further toward the hepatic lineage.* Stem Cells Dev, 2010. **19**(7): p. 961-78.
288. Soderdahl, T., et al., *Glutathione transferases in hepatocyte-like cells derived from human embryonic stem cells.* Toxicol In Vitro, 2007. **21**(5): p. 929-37.
289. Ek, M., et al., *Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells.* Biochem Pharmacol, 2007. **74**(3): p. 496-503.
290. McLean, A.B., et al., *Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed.* Stem Cells, 2007. **25**(1): p. 29-38.
291. D'Amour, K.A., et al., *Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells.* Nat Biotechnol, 2006. **24**(11): p. 1392-401.
292. Gouon-Evans, V., et al., *BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm.* Nat Biotechnol, 2006. **24**(11): p. 1402-11.
293. Gerlach, J.C., et al., *Interwoven four-compartment capillary membrane technology for three-dimensional perfusion with decentralized mass exchange to scale up embryonic stem cell culture.* Cells Tissues Organs, 2010. **192**(1): p. 39-49.
294. Serra, M., et al., *Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control.* J Biotechnol, 2010. **148**(4): p. 208-15.
295. Baharvand, H., et al., *Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro.* Int J Dev Biol, 2006. **50**(7): p. 645-52.
296. Miki, T., A. Ring, and J. Gerlach, *Hepatic differentiation of human embryonic stem cells is promoted by three-dimensional dynamic perfusion culture conditions.* Tissue Eng Part C Methods, 2011. **17**(5): p. 557-68.
297. Ring, A., et al., *Hepatic maturation of human fetal hepatocytes in four-compartment three-dimensional perfusion culture.* Tissue Eng Part C Methods, 2010. **16**(5): p. 835-45.
298. Hay, D.C., et al., *Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo.* Stem Cells, 2008. **26**(4): p. 894-902.
299. Hay, D.C., et al., *Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities.* Cloning Stem Cells, 2007. **9**(1): p. 51-62.
300. Cable, E.E. and H.C. Isom, *Exposure of primary rat hepatocytes in long-term DMSO culture to selected transition metals induces hepatocyte proliferation and formation of duct-like structures.* Hepatology, 1997. **26**(6): p. 1444-57.

301. Su, T. and D.J. Waxman, *Impact of dimethyl sulfoxide on expression of nuclear receptors and drug-inducible cytochromes P450 in primary rat hepatocytes.* Arch Biochem Biophys, 2004. **424**(2): p. 226-34.
302. Kidambi, S., et al., *Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance.* Proc Natl Acad Sci U S A, 2009. **106**(37): p. 15714-9.
303. Hay, D.C., et al., *Unbiased screening of polymer libraries to define novel substrates for functional hepatocytes with inducible drug metabolism.* Stem Cell Res, 2011. **6**(2): p. 92-102.
304. Schuldiner, M., et al., *Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11307-12.
305. Itskovitz-Eldor, J., et al., *Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers.* Mol Med, 2000. **6**(2): p. 88-95.
306. Shiraki, N., et al., *Differentiation of mouse and human embryonic stem cells into hepatic lineages.* Genes Cells, 2008. **13**(7): p. 731-46.
307. Lazaro, C.A., et al., *Generation of hepatocytes from oval cell precursors in culture.* Cancer Res, 1998. **58**(23): p. 5514-22.
308. Soto-Gutierrez, A., et al., *Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines.* Nat Protoc, 2007. **2**(2): p. 347-56.
309. Takayama, K., et al., *Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1alpha transduction.* J Hepatol, 2012.
310. Sekiya, S. and A. Suzuki, *Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors.* Nature, 2011. **475**(7356): p. 390-3.
311. Huang, P., et al., *Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors.* Nature, 2011. **475**(7356): p. 386-9.
312. George, J., et al., *Time-dependent expression of cytochrome P450 genes in primary cultures of well-differentiated human hepatocytes.* J Lab Clin Med, 1997. **129**(6): p. 638-48.
313. Lopez-Garcia, M.P., *Endogenous nitric oxide is responsible for the early loss of P450 in cultured rat hepatocytes.* FEBS Lett, 1998. **438**(3): p. 145-9.
314. Sidhu, J.S., F. Liu, and C.J. Omiecinski, *Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: requirement of dexamethasone and extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes.* Exp Cell Res, 2004. **292**(2): p. 252-64.
315. Gomez-Lechon, M.J., et al., *Expression and induction of a large set of drug-metabolizing enzymes by the highly differentiated human hepatoma cell line BC2.* Eur J Biochem, 2001. **268**(5): p. 1448-59.
316. Butura, A., et al., *Differentiation of human hepatoma cells during confluence as revealed by gene expression profiling.* Biochem Pharmacol, 2004. **67**(7): p. 1249-58.
317. Favre, C., et al., *Putrescine decreases cytochrome P450 3A4 levels during liver regeneration in the rat.* J Hepatol, 1998. **28**(4): p. 700-8.
318. Pondugula, S.R., et al., *Protein phosphatase 2Cbetal regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells.* Drug Metab Dispos, 2010. **38**(9): p. 1411-6.
319. Rochette-Egly, C., *Nuclear receptors: integration of multiple signalling pathways through phosphorylation.* Cell Signal, 2003. **15**(4): p. 355-66.
320. Lin, W., et al., *Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells.* J Biol Chem, 2008. **283**(45): p. 30650-7.
321. Sugatani, J., et al., *Regulation of PXR Function and UGT1A1 Gene Expression by Post-translational Modification of PXR Protein.* Drug Metab Dispos, 2012.
322. Cheng, A., et al., *Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases.* Genes Dev, 1999. **13**(22): p. 2946-57.

323. Hino, H., et al., *A long-term culture of human hepatocytes which show a high growth potential and express their differentiated phenotypes.* Biochem Biophys Res Commun, 1999. **256**(1): p. 184-91.
324. Choi, S., et al., *Characterization of increased drug metabolism activity in dimethyl sulfoxide (DMSO)-treated Huh7 hepatoma cells.* Xenobiotica, 2009. **39**(3): p. 205-17.
325. Iwase, M., et al., *Evaluation of the effects of hydrophilic organic solvents on CYP3A-mediated drug-drug interaction in vitro.* Hum Exp Toxicol, 2006. **25**(12): p. 715-21.
326. Chauret, N., A. Gauthier, and D.A. Nicoll-Griffith, *Effect of common organic solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes.* Drug Metab Dispos, 1998. **26**(1): p. 1-4.
327. Makowske, M., et al., *Immunochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid.* J Biol Chem, 1988. **263**(7): p. 3402-10.
328. Leiter, J.M., W. Helliger, and B. Puschendorf, *Increase in histone acetylation and transitions in histone variants during Friend cell differentiation.* Exp Cell Res, 1984. **155**(1): p. 222-31.
329. Fiore, M. and F. Degrassi, *Dimethyl sulfoxide restores contact inhibition-induced growth arrest and inhibits cell density-dependent apoptosis in hamster cells.* Exp Cell Res, 1999. **251**(1): p. 102-10.
330. Mancone, C., et al., *Proteomic analysis reveals a major role for contact inhibition in the terminal differentiation of hepatocytes.* J Hepatol, 2010. **52**(2): p. 234-43.
331. Roth, R.A., et al., *Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents?* Toxicol Appl Pharmacol, 1997. **147**(2): p. 300-11.
332. Ganey, P.E., et al., *Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor.* Chem Biol Interact, 2004. **150**(1): p. 35-51.
333. Dhawan, A., et al., *Human hepatocyte transplantation.* Methods Mol Biol, 2010. **640**: p. 525-34.
334. Fox, I.J., et al., *Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation.* N Engl J Med, 1998. **338**(20): p. 1422-6.
335. Schneider, A., et al., *Hepatocyte transplantation in an acute liver failure due to mushroom poisoning.* Transplantation, 2006. **82**(8): p. 1115-6.
336. Ben-David, U. and N. Benvenisty, *The tumorigenicity of human embryonic and induced pluripotent stem cells.* Nat Rev Cancer, 2011. **11**(4): p. 268-77.
337. Eilers, M. and R.N. Eisenman, *Myc's broad reach.* Genes Dev, 2008. **22**(20): p. 2755-66.
338. Li, Y., et al., *Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4.* Blood, 2005. **105**(2): p. 635-7.
339. Sato, N., et al., *Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor.* Nat Med, 2004. **10**(1): p. 55-63.