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CELLULAR REPLACEMENT THERAPY FOR LIVER DISEASE

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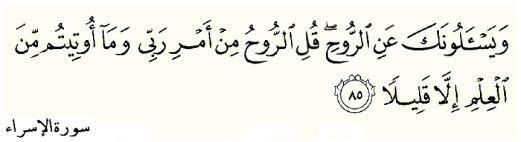
وَيَسْـَٔلُونَكَ عَنِ ٱلرُّوحِ ۖ قُلِ ٱلرُّوحُ مِنْ أَمْرِ رَبِّى وَمَآ أُوتِيتُم مِّنَ ٱلْعِلْمِ إِلَّا قَلِيلًا ﴿٨٥﴾

سورة الإسراء

And they ask you, [O Muhammad], about the soul. Say, "The soul is of the affair of my Lord. ***And mankind have not been given of knowledge except a little****." Quran 17:85*

To my parents with love

ABSTRACT

Liver disease is a major health problem worldwide. The liver performs a wide range of functions, which the human body cannot survive without. The human liver is continuously challenged with infectious organisms, alcohol and chemicals, congenital defects, autoimmunity and malignancy etc. The liver has been imparted a marvelous capacity to regenerate and recover from various insults. However, in many cases liver injuries exceed its regenerative capacity with end-stage liver disease becoming the inevitable end.

So far, liver transplantation is still the only treatment modality for end-stage liver disease. However, there are many limitations to liver transplantation towards its applicability and availability for all patients worldwide, such as scarcity of donors as well as other ethical, technical and surgical considerations.

Cell transplantation is a frequently studied alternative to organ transplantation in liver disease. Many cell types are under extensive evaluation, with primary human hepatocytes and different stem cell types coming first on the list. For primary human hepatocytes, liver tissue is still needed, and when available, cells are produced in huge numbers requiring cryopreservation. Available hepatocyte cryopreservation protocols still need further optimization. In addition, better cold storage techniques for hepatocytes are needed for the feasibility of frequent cell infusions per patient. Stem cells still need to be studied further for their differentiation potential towards hepatic lineages, safety, immunomodulatory roles, and their possible support for co-transplanted hepatocytes.

In this thesis, we addressed a few of the current obstacles facing cellular replacement therapy for liver disease. In the first study, we isolated and characterized a mesenchymal stem cell population from human fetal liver. The hepatic origin, the mesenchymal nature, and the immunomodulatory effects of these cells suggest them as potential candidates for cellular therapy for liver disease. In addition, we transplanted these cells into a mouse model of liver disease with an evidence for their potential differentiation to hepatocyte-like cells *in vivo*. In the second study, we characterized microRNAs expressed in the human liver. Such information can help understanding the role of microRNAs in liver development and their potential use in microRNA-based stem cell differentiation towards hepatic lineages. In the third study, we introduced a new defined xeno-free cryoprotectant to the field of hepatocyte cryopreservation. This cryoprotectant could be of value when preserving hepatocytes and stem cell-derived hepatocytes in a clinical setting. In the fourth study, we showed that human liver material could be better cold-stored as a whole tissue rather than as single cells. This makes it possible for frequent hepatocyte infusions commonly needed in a clinical context.

LIST OF PUBLICATIONS

I. ***Saliem M**, Ericzon BG, Ellis E, Hovatta O, Götherström C (2012).*
Isolation and Characterization of Mesenchymal Stem Cells from Human Fetal Liver; Potential Candidates for Replacement Therapy in Liver Disease.
J Liver: Dis Transplant 1:2. doi:10.4172/jldt.1000102

II. ***Saliem M**, Zhao Y, Greco D, Kere J, Ellis E, Hovatta O, Asikainen S.*
The Role of MicroRNAs in Hepatocyte Development and Hematopoiesis in Human Fetal and Adult Livers.
Manuscript in revision

III. ***Saliem M**, Holm F, Tengzelius RB, Jorns C, Nilsson LM, Ericzon BG, Ellis E, Hovatta O.*
Improved Cryopreservation of Human Hepatocytes Using a New Xeno-free Cryoprotectant Solution.
World J Hepatol. 2012 May 27;4(5):176-83.

IV. *Jorns C, Gramignoli R, **Saliem M**, Zemack H, Mörk LM, Isaksson B, Nowak G, Ericzon BG, Strom S and Ellis E.*
Strategies for Short-term Storage of Hepatocytes for Repeated Clinical Infusions.
Cell Transplant. 2012 Dec 8. [Epub ahead of print]

RELATED PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. *Al-Saqi SH, **Saliem M**, Asikainen S, Quezada HC, Le Blanc K, Jonasson AF, Götherström C.*
In vitro comparative study of adipose and bone marrow derived mesenchymal stem cells cultured in serum- and xeno-free versus serum-based medium.
Manuscript submitted

II. *Al-Saqi SH, **Saliem M**, Quezada HC, Le Blanc K, Jonasson AF, Hovatta O, Götherström C.*
Serum-and xeno-free cryopreservation of adipose-derived mesenchymal stem cells.
Manuscript submitted

CONTENTS

LIST OF ABBREVIATIONS

AFP	α-fetoprotein
ALB	albumin
BMP	bone morphogenetic protein
BNF	β-naphthoflavone
Calcein AM	calcein acetoxymethyl
CB	new cryoprotectant solution
CK	cytoskeletal protein
CRF	controlled rate freezing
CYP	cytochrome P450 enzyme
DME	drug metabolizing enzyme
DMEM-HG	high glucose Dulbecco's modified Eagle's medium
DMEM-LG	low glucose Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dsDNA	double stranded DNA
ESCs	embryonic stem cells
EGTA	ethylene glycol tetraacetic acid
EROD	ethoxyresorufin-O-deethylase
EthD-1	ethidium homodimer-1
FBS	fetal bovine serum
FGF	fibroblast growth factor
GFP	green fluorescent protein
HBSS	Hank's buffered salt solution
hFL-MSCs	human fetal liver mesenchymal stem cells
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HT	hepatocyte transplantation
ICC	immunocytochemical staining
iPSCs	induced pluripotent stem cells
LDA	live/dead assay
MSCs	mesenchymal stem cells
miRNA	microRNA
MLCs	mixed lymphocyte cultures
OLT	orthotopic liver transplantation
OSM	oncostatin M
PB	phenobarbital
PBLs	peripheral blood lymphocytes
PH	partial hepatectomy
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PSB	polystyrene box
RIF	rifampicin
RISCs	RNA-induced silencing complexes
TB	trypan blue exclusion method
UW	university of Wisconsin solution

1 INTRODUCTION

1.1 LIVER DISEASE AND MANAGEMENT OPTIONS

1.1.1 Global Burden of Chronic Liver Disease

Cirrhosis is the end stage for many liver diseases. It is characterized by liver fibrosis and nodular regeneration. This occurs as a result of a wound-healing response to chronic liver injury (Rockey and Friedman 2006). Common causes for cirrhosis are: viral infections such as with hepatitis B (HBV) and hepatitis C (HCV) infections, alcohol abuse, cholestasis, hepatotoxic drugs, nonalcoholic steatohepatitis (NASH), immune-mediated injury and genetic abnormalities. HBV infection is the most common cause in developing countries, while alcohol abuse, HCV infection and NASH are the most common in developed countries (Guha and Iredale 2007).

Up to 40% of patients with liver cirrhosis are asymptomatic as cirrhosis is often clinically silent (Falagas, Vardakas et al. 2007). This makes it difficult to estimate its prevalence and incidence accurately. However, based on autopsy studies, the prevalence of liver cirrhosis is 4.5% to 9.5% in the general population, which means that hundreds of millions of people are affected worldwide (Graudal, Leth et al. 1991).

The 5-year mortality in patients with liver failure is 50%; 70% of these are directly related to liver disease (Fattovich, Giustina et al. 1997). In 2001, 771,000 patients died from cirrhosis, and it was counted as the 14th leading cause of death worldwide accounting for 4.4% of all deaths (Mathers, Lopez et al. 2006). In 2020, it is expected to rise to the 12th position (Murray and Lopez 1997).

Chronic liver disease is the most common leading cause to hepatocellular carcinoma (HCC) (Fattovich, Stroffolini et al. 2004), which accounts for 70% to 85% of primary liver cancers in human (El-Serag 2001). Primary liver cancer represents about 5.6% of all human cancers (Parkin, Bray et al. 2001), and is expected to be higher in 2020 (Murray and Lopez 1997). Liver cancer is rapidly fatal with its incidence-to-mortality ratio is almost one (El-Serag 2001).

1.1.2 Current Management Options for Liver Disease

1.1.2.1 Liver Transplantation

Orthotopic liver transplantation (OLT) is still the only current treatment modality for end-stage liver disease and liver metabolic defects. When successful, the effectiveness of OLT is unquestionable. However, OLT has many limitations making the finding of other alternatives a necessity. First of all, organ donation has ethical limitations in certain communities, and because of high costs, OLT is not affordable in many areas of the world. Secondly, lack of donors is a major limitation with around 30% more patients on the waiting list than those who actually receive a liver, both in the USA and in Europe (Christ and Stock 2012). Also, every year up to 10% die while on the waiting list (Strom and Ellis 2011). Finally, OLT is not an ideal treatment for life-threatening liver emergencies such as acute liver failure. OLT is a major surgery requiring a significant recovery time and with high incidence of surgery related complications. As an allogeneic surgery, OLT necessitates lifelong immunosuppression with its own complications such as higher incidence of infections and/or malignancies (Perera, Mirza et al. 2009).

1.1.2.2 Hepatocyte Transplantation

Hepatocyte transplantation (HT) is not yet an established treatment option for liver disease, but is under extensive investigation. Compared to OLT, HT is a less costly and less invasive procedure with shorter recovery periods and fewer complications. Keeping the native liver makes HT an ideal treatment option for metabolic liver disease with otherwise stable liver functions where hepatocytes can be used directly or after genetic modification (Birraux, Wildhaber et al. 2010). Successfully cryopreserved hepatocytes make HT a true option for cell therapy for acute liver failure (Fitzpatrick, Mitry et al. 2009). HT has gained proof-of-concept in more than 30 clinical trials where a repopulation of 1-5% was able to correct, at least transiently, defects in liver function bridging patients to OLT (Fox, Chowdhury et al. 1998; Hughes, Mitry et al. 2012). Nevertheless, liver donation and immunosuppression are still needed in HT. Since there is no strong evidence suggesting a lifelong efficacy for HT in humans, other sources for hepatocytes such as xenotransplants (Nagata, Ito et al. 2003), immortalized hepatocytes (Kobayashi, Fujiwara et al. 2000), and stem cell-derived hepatocytes (Chen, Tseng et al. 2012) should be perused. However, a fully optimized hepatocyte cryopreservation protocol is still lacking for all these treatment options.

1.1.2.3 Bioartificial Extracorporeal Liver Support System

It has been shown that many of the essential metabolic functions of the liver such as protein synthesis, urea production, conjugation, and detoxification might be provided by isolated hepatocytes (Yarmush, Dunn et al. 1992). Extracorporeal liver support systems are an experimental treatment option aiming at providing cellular support for the liver in e.g. acute liver failure. Similar to a hemodialysis device, an extracorporeal liver support system is composed of a bioreactor containing numerous hollow fibers of semipermeable membranes loaded with human or porcine hepatocytes and perfused with patient's plasma or blood (Sgroi, Serre-Beinier et al. 2009).

1.2 LIVER STRUCTURE AND FUNCTION

1.2.1 Liver Structure

Liver is the largest gland in the body constituting around 2% of the adult body weight. Anatomically, the liver is composed of a parenchyma and stroma with parenchymal and non-parenchymal cells as well as vascular and biliary trees. The parenchyma comprises hepatocytes constituting the main part of the hepatic lobule. The non-parenchymal cells are mainly sinusoidal endothelial cells, Kupffer cells, stellate cells, dendritic cells and biliary epithelial cells. The liver tissue volume is composed as follows; 78% hepatocytes, 2.8% sinusoidal endothelial cells, 2.1% Kupffer cells, 1.4% hepatic stellate cells, with the extracellular space representing 16% (Gumucio, Berkovitz et al. 1996).

The hepatic lobule is the basic structural unit of the liver (Rappaport, Borowy et al. 1954). It has a characteristic hexagonal shape with liver cell plates radiating from the portal triads in the corners towards a central vein in the middle (figure 2.1). The liver cell plates are one-cell-thick hepatocyte cords. The hepatocyte plates pound hepatic sinusoids, which are lined by fenestrated sinusoidal endothelial cells. A portal triad contains interwoven branches of a portal vein, a hepatic artery and a bile duct. Blood

flows from both the portal vein and hepatic artery through the sinusoids ending in the central vein. The portal blood, coming mainly from the intestine, is rich in nutrients while the hepatic artery carries blood rich in oxygen (Ishibashi, Nakamura et al. 2009; Abdel-Misih and Bloomston 2010).

The hepatocytes are polarized epithelial cells. They perform their metabolic and endocrine functions through their basolateral surfaces across the fenestrated sinusoidal endothelium. Neighboring hepatocytes are interconnected with tight junctions circumscribing bile canaliculi facing the apical surface of the hepatocytes and through them hepatocytes perform their exocrine function, bile secretion (Si-Tayeb, Lemaigre et al. 2010).

Kupffer cells are resident liver tissue macrophages. They reside inside the sinusoids within gaps between the endothelial cells. They constitute the largest population of macrophages in the body and migrate to the site of injury within the liver (Ishibashi, Nakamura et al. 2009; Abdel-Misih and Bloomston 2010).

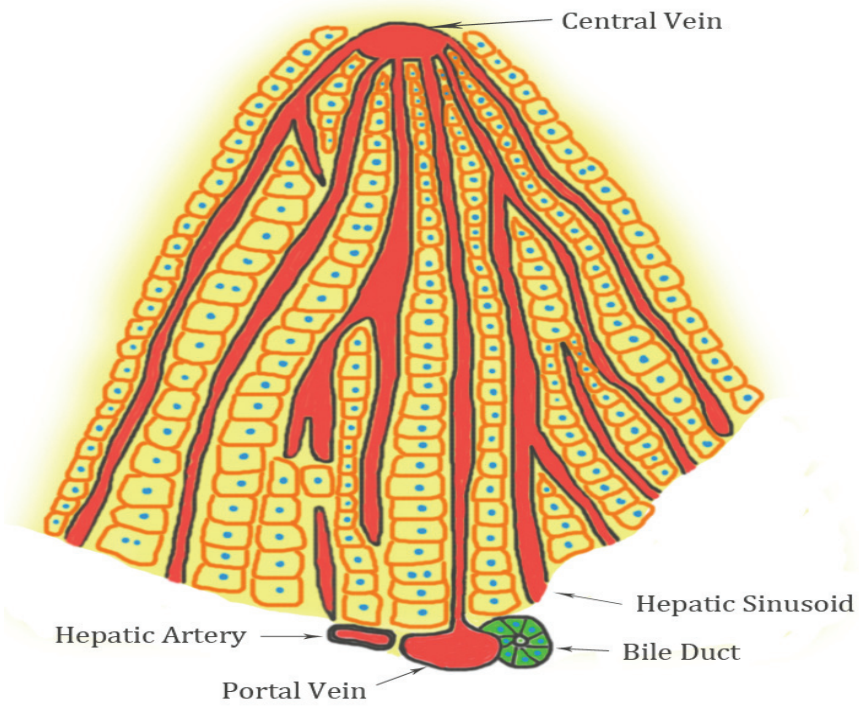

Figure 2.1: Microanatomy of a hepatic lobule. Hepatocytes are arranged in cords and are separated by hepatic sinusoids. Blood flows peripherally from the portal tract to the central vein through the sinusoids. The portal tract contains a branch of hepatic artery, a branch of portal vein and a bile duct. *Illustrated by Mohammed Saliem*.

Hepatic stellate cells reside in the perisinusoidal space of Disse and their main function is vitamin A storage. Upon liver injury, hepatic stellate cells transform into myofibroblasts producing extracellular matrix. As a result they play an important role in progression of fibrosis (Ishibashi, Nakamura et al. 2009; Abdel-Misih and Bloomston 2010).

1.2.2 Liver Function

Liver is a gland performing both exocrine and endocrine functions. The major exocrine function is bile secretion, which is essential for the absorption of fat and lipophilic nutrients. The endocrine functions are mainly secretion of many essential hormones e.g. thrombopoietin, angiotensinogen and insulin-like growth factor. The liver receives nutrients and transforms them into secreted proteins. Examples for these proteins are albumin, most of the clotting factors, the majority of plasma carrier proteins and apolipoproteins. The liver has also an essential role in drug metabolism and detoxification. The liver performs and controls many other metabolic processes such as glycogen storage, cholesterol synthesis and transport, as well as urea secretion and metabolism (Corless and Middleton 1983; Si-Tayeb, Lemaigre et al. 2010).
The fetal liver transiently also exhibits hematopoietic functions becoming the principle hematopoietic organ early in development. Hepatic progenitors and fetal liver stromal cells have been shown to support hematopoiesis *in vitro* (Hata, Nanno et al. 1993) (Martin and Bhatia 2005). On the other hand, differentiated hepatic progenitor cells did not support hematopoiesis (Kinoshita, Sekiguchi et al. 1999). Furthermore, hematopoietic cells from fetal liver expressed oncostatin M (OSM), which has been shown to enhance hepatocyte differentiation (Yoshimura, Ichihara et al. 1996). Together, these data suggest a dynamic interplay between hematopoietic, parenchymal and stromal cells within the developing fetal liver, controlling both hematopoiesis and hepatogenesis.

1.3 LIVER DEVELOPMENT

1.3.1 Competence, Induction and Specification

Upon gastrulation, three germ layers form: ectoderm, mesoderm and endoderm. The liver develops from the ventral part of the definitive endoderm delineating the primitive foregut. A crosstalk between three major neighboring areas is needed for proper development of the liver, one endodermal and two mesodermal: the gut endoderm, cardiogenic mesoderm and septum transversum mesenchyme (Tremblay and Zaret 2005) (figure 2.2A).
A widely accepted model for liver development begins when a certain group of endoderm cells gains 'competence' to differentiate toward hepatic lineages. 'Competent' endoderm cells are not specified yet, but gain the capacity to respond to liver specification-induction signals. Competence occurs when these cells start to express certain transcription factors, mainly forkhead box (FOX) A and GATA-binding protein 4 (GATA4) factors (Bossard and Zaret 1998). The expression of FOXA factors in the endoderm cells is depended, in turn, on their expression of hepatocyte nuclear factor (HNF) 1β (Lokmane, Haumaitre et al. 2008).
The next step in liver development is 'specification' where 'competent' endoderm cells start to respond to extracellular inducers specifying them to the hepatic fate (figure 2.2B). The first molecular evidence for hepatic specification is the expression of albumin, α-fetoprotein and transthyretin (Jung, Zheng et al. 1999). The first inducers to be identified are fibroblast growth factors (FGFs) originating from the cardiogenic mesoderm (Zaret 2008). A gradient concentration of FGFs is established and 'low' levels are crucial for the induction of hepatic genes. Later when more FGFs are produced by the cardiogenic mesoderm, their 'low' concentration in the hepatic domain

is maintained by specific spatial changes. The septum transversum grows intervening between the cardiogenic mesoderm and the pre-hepatic endoderm (Serls, Doherty et al. 2005). FGFs mediate their hepatic induction through the mitogen-activated protein kinase (MAPK) signaling pathway (Calmont, Wandzioch et al. 2006). In addition to FGFs, bone morphogenetic proteins (BMPs) from the septum transversum mesenchyme contribute to hepatic-specific gene induction, probably through stimulation of GATA4 expression (Shin, Shin et al. 2007). It has been shown that Wnt signaling also has an important role in hepatic induction. Early in development, repression of Wnt signaling anteriorly by endoderm is crucial for maintaining foregut identity and for allowing for liver development.

1.3.2 Formation of the Hepatic Bud

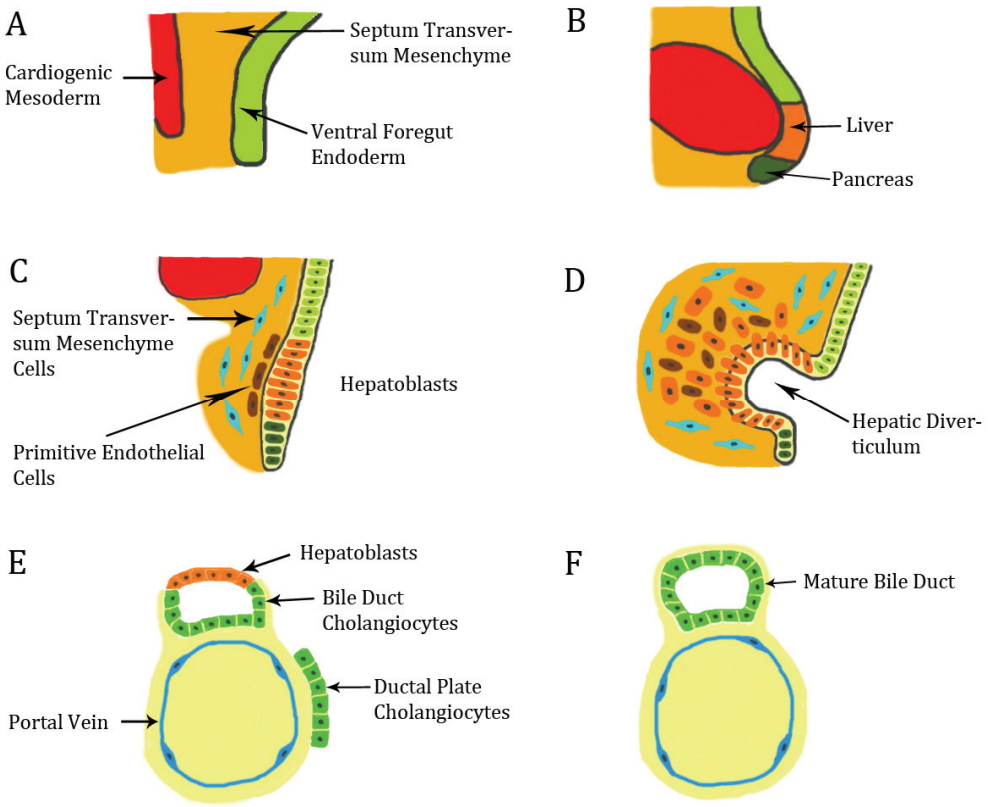

Figure 2.2: Liver development in human. An interaction between foregut endoderm, septum transversum mesenchyme and cardiogenic mesoderm is needed. Foregut endoderm first gains competence to develop to liver (A) and then becomes specified to liver fate (B). Hepatoblasts develop from specified endoderm and migrate into septum transversum mesenchyme (C). Hepatic diverticulum forms and developing liver buds into septum transversum mesenchyme (D). Bile duct formation starts with ductal plate development into asymmetric duct with hepatoblasts constituting part of its wall then matures by having only cholangiocytes in its wall (E&F). *Illustrated by Mohammed Saliem*.

The first gross morphological event in human liver development is formation of hepatic diverticulum by day 22 (figure 2.2C&D). 'Competent' endoderm cells, now called

hepatoblasts, bud into the surrounding septum transversum mesenchyme. Hepatoblasts are first columnar, and then become pseudostratified epithelium (Martinez Barbera, Clements et al. 2000). Hepatoblasts face a basement membrane, which separates them from an endothelial cell layer (figure 2.2C). The basement membrane contains fibronectin, laminin, collagen IV, nidogen and heparan sulfate proteoglycan (Bort, Signore et al. 2006).
A symphony of complex cellular events is involved in the formation of the hepatic bud. This includes cellular proliferation, migration, adhesion and differentiation. For migration, developing hepatoblasts undergo transient epithelial-mesenchymal transition and downregulation of E-cadherin expression. The homeobox (Hex) factor, GATA6, HNF6, Onecut (OC) 2, T-box transcription factor 3 (Tbx3), prospero-related homeobox-1 (Prox1) have been all shown to be involved in hepatoblast migration and adhesion (Lemaigre 2009).

1.3.3 Expansion of the Liver

Continuous interactions between hepatoblasts and adjacent mesodermal, endothelial and mesenchymal cells are necessary for the expansion of the liver and several growth factors are involved. Hepatocyte growth factor (HGF) is secreted by the septum transversum mesenchyme, the endothelial cells and the hepatoblasts. HGF acting on its receptor c-met on the hepatoblasts stimulates the SEK1/MKK4 pathway contributing to hepatoblast proliferation and liver expansion (Nishina, Vaz et al. 1999). The transforming growth factor (TGF) β-Smad2/Smad3 signaling pathway also is involved (Weinstein, Monga et al. 2001). Hepatoma-derived growth factor (HDGF) appears to stimulate proliferation of hepatoblasts *in vitro*; however this lacks an *in vivo* evidence suggesting unknown compensatory mechanisms to be involved (Gallitzendoerfer, Abouzied et al. 2008). It has also been shown that Wnt ligands, e.g. Wnt9, and their Frizzled receptors mediate hepatoblast proliferation (Matsumoto, Miki et al. 2008). Wnt signaling pathway, and its canonical mediator β-catenin, may help in achieving global liver morphology through localized control of hepatic cell proliferation creating localized growth zones in the liver (Suksaweang, Lin et al. 2004). Mesenchymal stem cells (MSCs) intermingled with hepatoblasts have been shown to stimulate hepatoblast proliferation by secreting hepatotrophic factors. The production of these trophic factors may be mediated by retinoic acid and its receptor RXRα expressed in MSCs. In addition to its indirect effect on hepatoblast proliferation, retinoic acid may also affect liver lobulation and global morphology (Ijpenberg, Perez-Pomares et al. 2007). Proper liver expansion is not only dependent on the stimulation and regional control of hepatoblast proliferation, but also on prevention of their apoptosis. It has been shown that expression of the transcription factor nuclear factor κB (NFκB) protects against apoptosis mediated by tumor necrosis factor (TNF) (Doi, Marino et al. 1999).

1.3.4 Fate Decision of Liver Cells

Cell fate decisions during liver development refer mainly to the commitment of hepatoblasts either to hepatocyte lineage or cholangiocyte lineage. However, it is also noteworthy to consider the development of other cellular components in the liver: vascular and sinusoidal endothelial cells, stellate cells and Kupffer cells. Cell fate decision during liver development is still not fully explained.

Explaining hepatobiliary lineage commitment is depended on the identification of hepatocyte- and cholangiocyte-specific markers. Many of these lineage-specific markers are expressed, to a lesser extent, in the other lineage or in the definitive endoderm. For example, the cholangiocyte-specific cytoskeletal (CK) proteins 7 and 19 are not the best indicators for biliary cell fate decision. For CK7, it is only detectable when the biliary cell fate decision has been already established. CK19 is also expressed in hepatocytes, albeit to a lesser degree (Van Eyken, Sciot et al. 1988). However, expression of the transcription factor hepatocyte nuclear factor 4α (HNF4α) is considered mandatory for hepatocyte commitment (Li, Ning et al. 2000). Similarly, the re-expression of the SRY-related HMG box transcription factor 9 (SOX9) is considered necessary for cholangiocyte commitment (Antoniou, Raynaud et al. 2009). There is some evidence indicating a role for Tbx3 (Ludtke, Christoffels et al. 2009) and HNF6 (Clotman, Lannoy et al. 2002) in the timing of hepatoblast fate decision, by promoting their biliary differentiation. TGFβ (Clotman, Jacquemin et al. 2005), Jagged-Notch (Tanimizu and Miyajima 2004), Wnt/β-catenin (Hussain, Sneddon et al. 2004), FGF2/7 (Yanai, Tatsumi et al. 2008), BMP4 (Ader, Norel et al. 2006) have potential roles in biliary differentiation and hepatoblast fate decisions. On the other hand, HGF promotes hepatocyte differentiation by stimulating the expression of the transcription factor CCAAT enhancer binding protein α (C/EBPα) (Suzuki, Iwama et al. 2003). Whether different extracellular matrix components influence hepatoblast fate determination still needs further evaluation after these controversial data.

Sinusoidal endothelial cells express the vascular adhesion molecule-1 (VAP1), stabilin 1 and 2, L-SIGN, reelm and have low expression of CD31 and von Willibrand factor, but do not express CD34 (type 1 transmembrane sialomucin), which is regularly expressed in other endothelial cell types (Nonaka, Tanaka et al. 2007). Also, they share expression of the lymphatic vascular endothelial hyaluronan receptor-1 (LYVE1) with lymphatic endothelial cells (Mouta Carreira, Nasser et al. 2001).

Hepatic stellate cells may have a mesothelial, rather than endodermal or mesenchymal origin. There is some evidence for a potential role for Wilm's Tumor (Wt1) and retinoic acid in the development of hepatic stellate cells (Ijpenberg, Perez-Pomares et al. 2007). Hepatic cells expressing the activated leukocyte cell adhesion molecule (ALCAM) have similar features to hepatic stellate cells (Loo and Wu 2008).

1.3.5 Development of Hepatic Vasculature

During liver development, new blood vessels form either *de novo* (vasculogenesis) or sprout out from preexisting vessels (angiogenesis). Hepatic sinusoids are the first part of the hepatic vascular network to develop. They form by angiogenesis from vessels of the septum transversum mesenchyme (Collardeau-Frachon and Scoazec 2008). There is some evidence for involvement of vasculogenesis with the formation of new endothelial cells of mesothelial origin (Perez-Pomares, Carmona et al. 2004). Hepatic sinusoids gradually mature both anatomically and functionally as their endothelial cells mature probably as a result of changes in extracellular matrix composition with development (Nonaka, Tanaka et al. 2007).

The efferent venous system of the fetal liver develops from the vitelline veins. The major efferent venous supply to the fetal liver comes from the umbilical vein. The portal vein replaces the umbilical vein after birth (Collardeau-Frachon and Scoazec 2008). The hepatic artery develops after the venous system along the intrahepatic portal

vein and gradually extends to the periphery (Gouysse, Couvelard et al. 2002). It has been postulated that development of the intrahepatic arterial system is directed by the developing ductal plate (see below) and its secreted vascular endothelial growth factor (VEGF) (Clotman, Libbrecht et al. 2003).

The intrahepatic bile ducts are formed by cholangiocytes developing from hepatoblasts. Cholangiocyte differentiation is stimulated by TGFβ signaling with higher ligand concentration closer to portal branches (Antoniou, Raynaud et al. 2009). There is some evidence suggesting the involvement also of Notch signaling in cholangiocyte development (McDaniell, Warthen et al. 2006). Notch signaling may help restrict cholangiocyte differentiation to periportal hepatoblasts (Zong, Panikkar et al. 2009). In addition to TGFβ and Notch, other signaling pathways have been suggested to contribute to cholangiocyte differentiation like Wnt (Decaens, Godard et al. 2008) as well as FGF and BMP (Yanai, Tatsumi et al. 2008). Cholangiocytes first arrange themselves as a monolayer ring called the ductal plate along the intrahepatic portal branches. A second layer of hepatoblasts arranges itself closer to the parenchymal side of the primary cholangiocyte layer separated by a luminal space and creating an asymmetrical ductal structure (figure 2.2E). This asymmetrical structure is transient as the second hepatoblast layer differentiates to cholangiocytes in a second wave of cholangiocyte development (figure 2.2F). In addition to their role in cholangiocyte differentiation, both Notch and TGF-β signaling pathways are instrumental in biliary duct formation (Antoniou, Raynaud et al. 2009). Similarly, several transcription factors are involved in both processes such as HNF1β (Coffinier, Gresh et al. 2002); HNF6 and Onecut2 (Clotman, Lannoy et al. 2002); as well as C/EBPα (Yamasaki, Sada et al. 2006) and Hhex (Hunter, Wilson et al. 2007). Towards the end of gestation, when cholangiocyte differentiation and duct symmetry are accomplished, cholangiocyte multiplication continues to add to biliary duct growth.

1.3.6 Hepatocyte Maturation

After hepatoblast fate determination, developing hepatocytes undergo maturation where the hepatocytes gradually acquire their characteristic morphology and functions. Hepatocyte maturation is a continuous process extending even to the neonatal period. Jaundice appears normally in neonates sometimes due to a physiological delay in the ability of hepatocytes to conjugate bilirubin (Kaniwa, Kurose et al. 2005). Maturation is effected by a dynamic transcriptional network with progressive increase in complexity (Petkov, Zavadil et al. 2004). Several liver-enriched factors such as HNF1α, HNF1β, FoxA2, HNF4α1, HNF6 and Liver receptor homolog-1 (LRH1) showed synergistic interdependence where they are able to mutually control each other and to cooperate with other factors in stimulating target gene expression (Kyrmizi, Hatzis et al. 2006). The progressive increase in concentration of these factors and the interplay between them throughout the maturation process may help activate liver-specific genes at certain time points during different developmental stages.

Several studies have linked many of the liver-enriched factors to certain liver functions. For example, HNF1α/4α were linked to glucose, lipid and amino acid metabolism (Odom, Zizlsperger et al. 2004); HNF1β to bile acid sensing and fatty acid oxidation (Coffinier, Gresh et al. 2002); HNF6 to growth hormone (Lahuna, Fernandez et al. 1997) and glucocorticoid activity (Pierreux, Stafford et al. 1999); LRH1 to bile acid and cholesterol metabolism (Lee and Moore 2008); C/EBPα to glucose, glycogen and

lipid metabolism and hepatocyte proliferation and C/EBPβ to gluconeogenesis (Lekstrom-Himes and Xanthopoulos 1998).

Gene expression is the main process involved in hepatocyte maturation. However, some genes need to be repressed for maturation to take place. For example, the proliferation factor hepatoma-derived growth factor (HDGF) is downregulated later in liver development (Enomoto, Yoshida et al. 2002). Similarly, α-fetoprotein is downregulated in adult liver when its gene is repressed by for example the zinc finger and homeoboxes factor 2 (zhx2) (Perincheri, Dingle et al. 2005) or the Zinc finger factor, zinc finger and BTB domain containing 20 (ZBTB20) (Xie, Zhang et al. 2008). It is noteworthy to mention that both zhx2 and ZBTB20 are not liver-specific proteins exerting liver-specific functions.

In addition to zhx2 and ZBTB20, oncostatin M (OSM) is necessary for hepatocyte maturation while coming from a non-hepatic origin. OSM is an interleukin-6-related cytokine secreted by hematopoietic cells abundantly available in the developing liver being the first hematopoietic organ in the body. OSM stimulated the expression of terminal hepatocyte differentiation markers through binding to gp130 receptor initiating a STAT3-mediated signaling cascade (Ito, Matsui et al. 2000). The transcription factor Jumonji also mediates OSM activity (Anzai, Kamiya et al. 2003).

The achievement of the characteristic cord-like arrangement of hepatocytes is effected by the guanosine triphosphatase adenosine diphosphate-ribosylation factor 6 (ARF6), which seems to be dependent on HGF in its function (Suzuki, Kanai et al. 2006). There is some evidence for a role for sinusoidal endothelial cells in establishing the apico-basal polarity in hepatocytes (Sakaguchi, Sadler et al. 2008). HGF is essential for hepatocyte maturation exerting its effects, similar to OSM, mainly postnatal as suggested from its higher neonatal levels (Kamiya, Kinoshita et al. 2001).

Hepatocytes undergo metabolic zonation where hepatocytes at different locations in the hepatic lobule have different metabolic functions. Hepatocytes are thus a heterogeneous cell population expressing different sets of genes between the periportal and the pericentral zones (Jungermann and Katz 1989). For example, glutamine synthetase, ornithine aminotransferase and thyroid hormone receptor β are exclusively expressed in the pericentral zone. There is some evidence indicating that periportal repression of glutamine synthetase is effected by histone deacetylase type I recruited by HNF4α (Stanulovic, Kyrmizi et al. 2007). Another example is the complementary expression of β-catenin and its repressor adenomatous polyposis coli protein (APC). APC is considered as the key regulator of lobular zonation. For APC being expressed periportal prevents pericentrally-expressed β-catenin from being expressed also periportal thus ensuring the periportal and pericentral gene expression patterns (Benhamouche, Decaens et al. 2006).

The metabolic zonation events take place mainly after birth (Jungermann and Katz 1989) reflecting the importance of the postnatal period in hepatocyte maturation. This is best illustrated by the ontogeny of liver drug metabolizing enzymes (DMEs), mainly the cytochrome P450 enzymes (CYPs) (See below).

1.3.7 Ontogeny of Liver Drug Metabolizing Enzymes

DME ontogeny refers to the time-wise expression of different DMEs during liver development (table 2.1). Although human liver starts to develop as early as the 4^{th} week of gestation, it is not before the 8^{th}-10^{th} weeks when the first DME activities are detected (Stevens, Hines et al. 2003). DMEs (Hines 2008; Hart, Cui et al. 2009) can be divided into 2 major groups: oxidative and conjugation enzymes. The oxidative group contains 5 major families: alcohol dehydrogenases (ADHs), aldehyde oxidases

(AOXs), CYPs, flavin-containing monooxygenases (FMOs) and paroxonases (PONs). The conjugation group contains 4 major families: epoxide hydrolases (EPHXs), glutathione S-transferases (GSTAs), sulfotransferases (SLUTs) and uridine 5'-diphosphoglucuronic acid (UDP) glucuronosyltransferases (UGTs).

ADHs include 7 major enzymes: ADH1A, -1B, -1C, ADH4, ADH5, ADH6, ADH7. ADHs catalyze oxidation of alcohols and reduction of aldehydes. ADH1 enzymes catalyze ethanol oxidation (Edenberg and Bosron 1997).

AOXs include 2 members AOX1 and AOX2. AOXs have important roles in metabolism of aldehyde-containing and N-heterocyclic drugs and xenobiotics. CYPs include 59 members belonging to 18 families and 42 subfamilies. CYP1-3 families alone include 23 enzymes and carry on the majority of drug and toxicant metabolism. CYP1 family has 3 major members CYP1A1, -1A2 and -1B1. CYP2 family has 4 major subfamilies: CYP2A containing mainly CYP2A6, -2A7 and -2A13: CYP2C containing mainly CYP2C8, -2C9, -2C18 and -2C19: CYP2D6: and CYP2E1. CYP3 family includes mainly the CYP3A subfamily, which contains mainly CYP3A4, -3A5, -3A7 and -3A43 (Hines 2008).

CYP2C family constitutes 18% of CYPs in adult liver (Shimada, Yamazaki et al. 1994). They are responsible for around 29% of oxidative metabolism of clinically relevant drugs e.g. warfarin, phenytoin, diclofenac, ibuprofen, omeprazole and losartan (Williams, Hyland et al. 2004). CYP2D6 constitutes less than 2% of adult liver CYPs (Shimada, Yamazaki et al. 1994). It is responsible for oxidative metabolism of 12% of clinically relevant drugs e.g. atomoxetine as well as o-demethylation of codeine to morphine and of dextromethorphan to dextrorphan. CYP2E1 constitutes 7% of adult liver CYPs (Shimada, Yamazaki et al. 1994). It is responsible for oxidative metabolism of 2.5% of clinically relevant drugs e.g. acetaminophen, halothane and chlorzoxazone (Williams, Hyland et al. 2004) and bioactivation of e.g. ethanol, benzene and toluene (Tanaka, Terada et al. 2000).

CYP3A4, CYP3A5 and CYP3A7 are the most abundant liver CYPs while CYP3A34 is the least important isoform. They are responsible for oxidative metabolism of 46% of clinically-relevant drugs (Williams, Hyland et al. 2004). CYP3A4, alone, accounts for 10-50% of adult liver CYPs (Shimada, Yamazaki et al. 1994). CYP3A5 can exceed CYP3A4, depending on the presence of CYP3A5*1 allele) (Lin, Dowling et al. 2002). CYP3A7 is the dominant CYP3A isoform in fetal liver and accounts for 10-40% of adult CYPs, depending on the presence of 3A7*1C allele (Sim, Edwards et al. 2005).

FMO family includes 11 members FMO1-5 and 6P-11P. FMO1, FMO2 and FMO3 showed xenobiotic metabolic activities for e.g. tamoxifen, itopride, benzydamine, olopatidine and xanomeline (Krueger and Williams 2005).

PON1 is involved in metabolism and clearance of oxidized lipids (Durrington, Mackness et al. 2001) and some organophosphorous compounds e.g. soman, sarin and diazoxon (Costa, Li et al. 1999).

EPHXs exist mainly in 2 forms; microsomal (EPHX1) and soluble (EPHX2). EPHXs detoxify highly reactive xenobiotic epoxides (cyclic ethers) by adding water (hydrolase) to form dihydrodiols (Morisseau and Hammock 2005).

Enzyme	Ontogeny	Activity/Expression
ADH1A	Week 13.5	Dominant fetal form of ADH1s until week 36
ADH1B	Week 16	10% of adult values
ADH1C	Week 19	10% of adult values
AOXs	Neonatal	10-15% of adult values
CYP1A1	Week 7	Occasional expression in the adult
CYP1A2	Neonatal	4-5% of adult values
CYP2A6	Week 17 postnatal	Higher in the adult
CYP2C9	Weeks 8-22	1% adult values
CYP2C19	Weeks 12-40	10-20% of adult values
CYP2D6	Weeks 25-30	5% of adult values (in 30% of samples)
CYP2E1	Perinatal	10% of adult values (neonatal)
CYP3A7	Weeks 6-12	Mainly fetal (100-fold>CYP3A4)
CYP3A4	Weeks 7-17	10% of adult values (before week 30)
CYP3A5	Weeks 6-12	No age-related change
FMO1	Weeks 8-15	Mainly fetal
FMO3	First trimester (15% of samples)	Reaches adult values by year 18
PON1	Neonatal	Reaches a plateau 10-25 months postnatal
EPHX1	Weeks 7.6-21.9	50% of adult values
EPHX2	Weeks 14-27	20% of adult values
GSTM	Weeks 14-25	2.5-16.5% of total GST activity
GSTA	Weeks 14-25	45-90% of total GST activity
GSTP	Weeks 14-25	Mainly fetal (30-50% of total GST activity)
SULT1A1	Weeks 14-27	Fixed levels until 12 month postnatal
SULT1A3	Weeks 10-22	10-fold reduction postnatal
SULT1E1	First trimester	Progressive reduction afterwards
SULT1C2	Prenatal	Higher in adult
SULT2A1	Weeks 8-40	Reaches a plateau 3 months postnatal
UGT1A1	Months 3-6 postnatal	Adult values
UGT1A3	Prenatal	30% of adult values
UGT1A6	Prenatal	1-10% of adult values
UGT2B7	Weeks 15-27	10-20% of adult values
UGT2B17	Prenatal	<10% of adult values

Table 2.1: Ontogeny of the most common hepatic drug metabolizing enzymes (DMEs). *Summarized from (Hines 2008; Hart, Cui et al. 2009)*.

GSTs have three families cytosolic, microsomal and mitochondrial. GSTs help detoxify hydrophobic xenobiotics (containing carbon, nitrogen or sulfur atoms) by catalyzing the attacking by reduced glutathione. Cytosolic GSTs are considered as the most important among all GSTs for their role in drug and toxicant metabolism (Hayes,

Flanagan et al. 2005). Cytosolic GSTs include 16 members distributed into 6 subfamilies; GSTA (alpha), GSTM (mu), GSTO (omega), GSTP (pi), GSTT (theta) and GSTZ (zeta) (Nebert and Vasiliou 2004).
SULTs include 3 families, phenol SULTs (SULT1), hydroxysteroid SULTs (SULT2) and brain-specific SULT (SULT4). SULT1s include 4 subfamilies with 7 members; SULT1A1, -1A2, -1A3/4, -1B1, -1C2, -1C4 and -1E1. SULT2s have 2 subfamilies with 2 members; SULT2A1 and -2B1. SULT4 subfamily has only one member; SULT4A1 (Blanchard, Freimuth et al. 2004).
UGTs include 2 families with 16 genes. UGT1 family has 9 members; UGT1A1, -1A3, -1A4, -1A5, -1A6, -1A7, -1A8, -1A9 and -1A10 (Tukey and Strassburg 2000). UGT1A1 is responsible for bilirubin glucuronidation (de Wildt, Kearns et al. 1999).
UGT2 family has 7 members; UGT2A1, -2B4, -2B7, -2B10, -2B11, -2B15 and -2B17 (Tukey and Strassburg 2000). UGT2B7 is responsible for morphine glucuronidation (de Wildt, Kearns et al. 1999).

1.4 MICRORNAS

1.4.1 History and Formation

MicroRNAs (miRNAs) are the most abundant among the three major classes of small RNAs; miRNAs, small-interfering RNAs and Piwi-interacting RNAs. The first miRNA to be discovered was lin4 in *Caenorhabditis elegans* in 1993 (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993). Lin4 was discovered as a short non-protein coding transcript that was able to regulate the lin14 gene post-transcriptionally through its 3´ untranslated region (UTR). Seven years later, the miRNA let7 was discovered in a similar way to lin4 (Reinhart, Slack et al. 2000) and was found to be evolutionary conserved from insects to humans (Pasquinelli, Reinhart et al. 2000). This breakthrough triggered a revolution in miRNAs research, which began with the discovery of the let7 family that share the same sequence identity with let7 called 'seed' areas and have, as a result, similar mRNA targets (Kaufman and Miska 2010). Today, thousands of miRNAs have been discovered in humans and many other species, and their potential roles in health and disease are being unraveled.
miRNAs are first synthesized within the nucleus by RNA polymerase II/III as primary (pri)-miRNAs (Lee, Kim et al. 2004; Borchert, Lanier et al. 2006) (figure 2.3). Pri-miRNAs are long transcripts with a cap at the 5´end and a poly-adenine tail at the 3´end. Pri-miRNAs are processed by the nuclear Microprocessor complex, which contains RNase III Drosha and DGCR8/Pasha proteins, to produce precursor (pre)-miRNAs of 60-110 nucleotides in length. With the help of Exportin 5, Pre-miRNAs are exported to the cytoplasm to be processed further by RNase III Dicer-1 together with TRBP/PACT protein. The outproduct is imperfect double-stranded duplexes that need to be unwounded by helicase to form mature single-stranded miRNAs (Siomi and Siomi 2010).
Mature miRNAs are small RNAs of about 20-24 nucleotides in length. They work on the post-transcriptional level to regulate gene expression, using mechanisms which are not fully explained. What is known is that they bind to certain RNA-induced silencing complexes (RISCs) and guide them towards targeted genetic messages with fully or partially complementary sequences mainly in their 3´ UTRs (Brodersen and Voinnet 2009). The targeted messages are commonly repressed as a result of the miRNA-RISC action. It is not clearly understood how the repression happens but possible

explanations are; inhibition of mRNA translation into protein, accelerated mRNA decay or site-specific slicing in miRNA-mRNA pairs (Eulalio, Huntzinger et al. 2008). miRNAs use imperfect Watson-Crick base-pairing for mRNA target recognition. Nucleotides 2-7, called the 'seed' of miRNAs, are of special importance. Imperfect miRNA-mRNA hybrids with central bulges result in either translational inhibition or exonucleolytic mRNA decay, while highly complementary hybrids with central pairing result in mRNA slicing (Brodersen and Voinnet 2009).

The regulatory output of miRNA-mRNA interactions is dependent on many factors. The most important factors are: the complementary sites in targeted messages and the degree of their complementarity especially for the 'seed' regions; complementary site multiplicity; target site accessibility; the relative *in vivo* concentration of miRNAs to their target mRNAs; and the type of RISCs or Ago proteins on which miRNAs are loaded. Target site accessibility can be influenced by the presence of stable RNA secondary structures or RNA-binding proteins (Brodersen and Voinnet 2009).

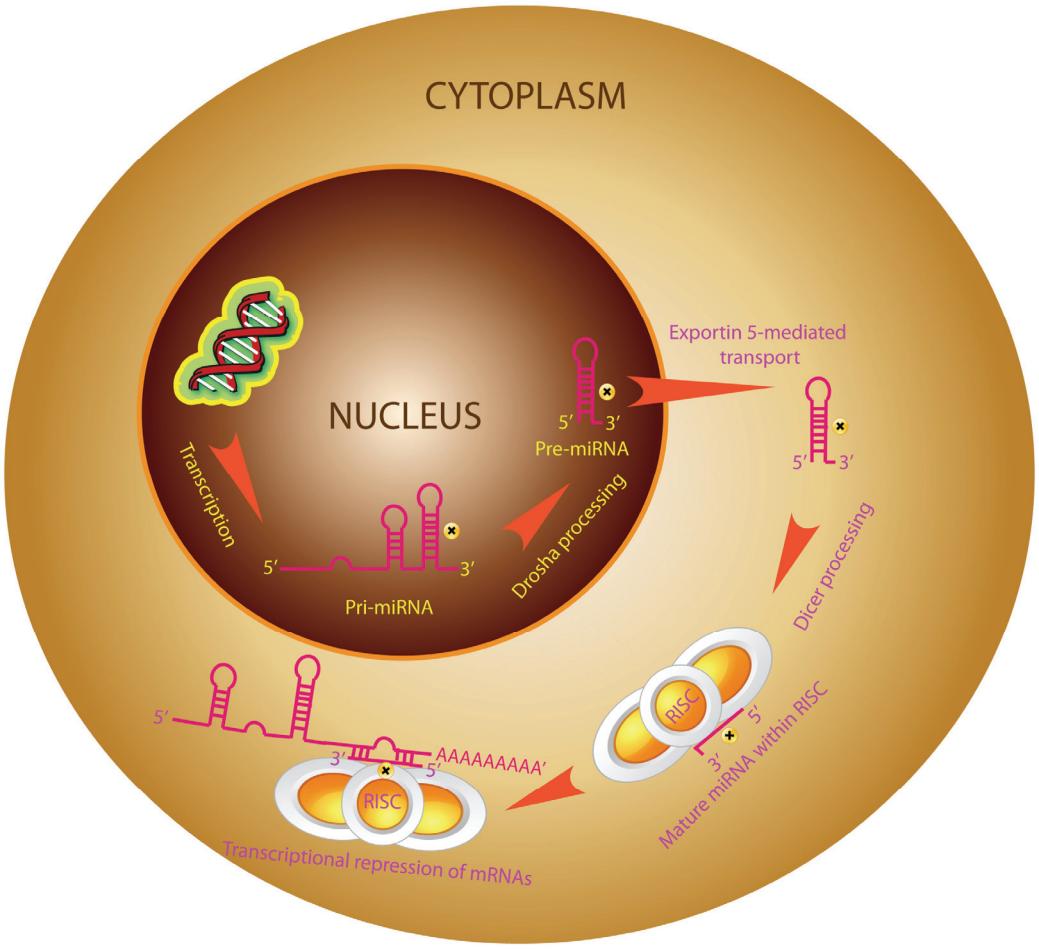

Figure 2.3: MicroRNA (miRNA) formation. miRNAs are transcribed in the nucleus as pri-miRNAs that are cleaved by Drosha to form pre-miRNAs, which are then exported to the cytoplasm and finally processed by Dicer producing mature miRNAs. Mature miRNAs bind to RISCs to modulate mRNA translation. *Illustrated by Mohammed Saliem*.

1.4.2 Potential Roles in Liver Development and Regeneration

miRNAs regulate about 30% of the protein-coding transcriptome (Filipowicz, Bhattacharyya et al. 2008) mainly through translational inhibition, or less commonly through translational activation (Vasudevan, Tong et al. 2007). miRNAs play an essential role in development, stress adaptation and hormone signaling (Leung and Sharp 2007). There is a growing interest for unraveling the potential roles for miRNAs during development. miRNAs have been shown to play key roles in cellular differentiation and fate determination (Yi, Poy et al. 2008). This can be due to targeting; transcription factors (Johnnidis, Harris et al. 2008), growth factor receptors (Eberhart, He et al. 2008), transcriptional repressors (Chen, Mandel et al. 2006) or proto-oncogenes (Xiao, Calado et al. 2007).

Until now, up to 42 human liver-specific miRNAs and their target genes have been identified. Some examples are miR-122, miR-148a, miR-192 and miR-194. These liver-specific miRNAs showed higher levels of expression in the fetal than the adult liver samples (Liu, Fan et al. 2010). Their levels of expression in the fetal samples showed dynamic changes between different developmental stages, suggesting a possible role for miRNAs in fetal liver development (Liu, Fan et al. 2009; Tzur, Israel et al. 2009; Liu, Fan et al. 2010). Some of the miRNAs had multiple variants and were expressed in a synergetic manner during fetal liver development (Liu, Fan et al. 2009). miRNA expression levels were inversely correlated to the expression levels of the putative target genes (Tzur, Israel et al. 2009).

Potential roles for miRNAs in liver development were further supported by Dicer deletion and miR-122 knockdown studies in mice. miR-122 is the most abundant among liver-specific miRNAs and miRNA depleted livers did not seem to be grossly affected up to 3 weeks postnatal. However, most of miRNA-deficient hepatocytes underwent early apoptosis and surviving animals had an increased incidence of hepatocellular carcinoma after 6 months (Esau, Davis et al. 2006; Elmen, Lindow et al. 2008; Sekine, Ogawa et al. 2009).

Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) showed a capacity to differentiate to hepatocytes. It is believed that miRNAs have important roles in stem cell differentiation. For example, miR-145 was suggested to downregulate the ESC pluripotency markers; octamer-binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2) and Kruppel-like factor 4 (KLF4) (Xu, Papagiannakopoulos et al. 2009). Also, miR-302 was suggested to promote ESC mesodermal differentiation by inhibiting lymphoid enhancer-binding factor (LEF) 1 and LEF2 expression (Rosa, Spagnoli et al. 2009).

Full understanding for the different roles that miRNAs might play during ESC/iPSC differentiation to hepatocytes is still lacking. Upon ESC endodermal induction *in vitro*, targets for the two most upregulated miRNAs were shown to inhibit endodermal differentiation (Tzur, Levy et al. 2008). In a very interesting work published recently, miRNA expression profiles were studied during ESC differentiation to hepatocytes. In this study, miRNA expression patterns showed dynamic changes between ESCs, ESC-derived endodermal cells and ESC-derived hepatocyte-like cells. In addition, miRNA profile for ESC-derived hepatocyte-like cells was similar to that for primary hepatocytes (Kim, Kim et al. 2011).

After partial hepatectomy (PH), extensive miRNA profiling identified seven highly upregulated miRNAs, miR-21 was of particular interest. It was suggested that miR-21

facilitates efficient cell cycle progression by antagonizing Btg2, a gene restraining G1-to-S transition phase (Song, Sharma et al. 2010).

1.5 LIVER REGENERATION

Due to its many essential functions, the liver is imparted a phenomenal capacity to regenerate. Liver regeneration was depicted in ancient Greek mythology by sinful Prometheus, whose liver was preyed by an eagle then renewed as fast as it was devoured providing him with eternal torture. Regeneration is a misnomer as resected tissue never grows back, but it is the remaining hepatocytes that compensate for the loss (Michalopoulos and DeFrances 1997). Hepatocytes have a tremendous replicative potential *in vivo* since only a few remaining hepatocytes can restore liver mass after profound injury (Overturf, Al-Dhalimy et al. 1999). Hepatocytes are normally long-lived resting in G0 phase of the cell cycle and rarely undergo mitosis (Michalopoulos and DeFrances 1997). Upon injury, up to 95% of quiescent hepatocytes rapidly re-enter the cell cycle with an increase in DNA synthesis noticed about 12 hours later. Non-parenchymal cells also re-enter cell cycle 2-3 days after hepatocytes (Stocker and Heine 1971). DNA synthesis begins first in cells surrounding the portal vein streaming towards the central vein (Rabes, Wirsching et al. 1976), and liver mass restoration is achieved within 5-7 days (Grisham 1962). The regenerative response is proportional to the degree of loss in liver tissue with restoring minimal losses even less than 10%. Liver tissue mass in relation to body size is crucial with regeneration stopping after mass restoration. Interestingly, livers transplanted from larger- to smaller-sized animals underwent reduction in their mass to restore an optimum liver mass/body mass ratio (Francavilla, Ove et al. 1988).

Strong evidence suggests the involvement of liver progenitor cells, e.g. SOX9 expressing, in the restoration of liver mass rather than mature cells. SOX9 expressing liver progenitors from bile ducts differentiate to hepatocytes in a wave streaming from the portal vein towards the central vein restoring lost hepatocytes (Furuyama, Kawaguchi et al. 2011). Also, mesenchymal or stellate cells can give rise to hepatocyte-like cells according to the mesenchymal-to-epithelial transition hypothesis (Choi and Diehl 2009).

Liver regeneration implicates many genetic and metabolic events that need to be switched on and off properly. Liver regeneration passes through three different phases. The first phase is called ‘priming phase’ where transcription of early hepatocyte genes is induced. Transcribed genes, such as *c-fos*, *c-jun* and *c-myc* help promote transition of quiescent hepatocytes from G0 phase to G1 phase. The second phase is the metabolic and growth factor phase where an increase in metabolic demands on the remnant liver takes place. The third phase is the termination phase where stop signals suppress regeneration once liver mass restoration is achieved. Proteins from TGF-β family are suggested to have a role in termination of regeneration (Romero-Gallo, Sozmen et al. 2005).

Restoration of liver histology after regeneration is not well explained. Two-three days after PH, newly formed hepatocytes arrange themselves into small clumps around blood capillaries. Hepatic stellate cells then send their processes penetrating through hepatocyte clumps secreting laminins. Capillaries transform into true hepatic sinusoids restoring the typical relationship between hepatocytes, sinusoidal endothelial cells and Kupffer cells. The high laminin content of the extracellular matrix is changed to a

mature form with the introduction of other proteins such as fibronectin, collagen I and collagen IV (Martinez-Hernandez and Amenta 1995). By the end of the first week, typical hepatic histology is restored but with larger lobules and two cell-layered hepatic plates (Ogawa, Medline et al. 1979).

However, liver regeneration is defective in many liver diseases. During cirrhosis there is a marked reduction in hepatocyte proliferative capacity with apparent failure in restoring normal liver histology. Defective hepatocyte proliferation could be due to expression of hepatocyte nuclear p21 and the progressive telomere shortening (Paradis, Youssef et al. 2001; Wiemann, Satyanarayana et al. 2002). There is strong evidence suggesting that regenerative nodules in cirrhotic liver originate from liver progenitor cells rather than from mature hepatocytes (Lin, Lim et al. 2010). Liver regeneration is also impaired in obesity and NASH (Farrell, Robertson et al. 2002).

1.6 STEM CELL THERAPY FOR LIVER DISEASE

Stem cells are defined as undifferentiated cells capable of; high proliferation, self-maintenance, production of differentiated functional progeny and regenerating their tissues of origin (Potten and Loeffler 1990). The main functions of stem cells are to produce the whole organism during development, and to function as an internal repair system in mature tissues and organs. Thus, stem cells carry great promise in regenerative medicine to repair damaged tissues and organs. Based on their origin and their role in development or tissue repair, stem cells can be divided as ESCs and non-embryonic (adult, somatic or tissue-specific stem cells such as MSCs). In 2006, a third stem cell type, iPSCs, was introduced when scientists succeeded to genetically reprogram mature cells to assume a stem cell-like state.

We learned from liver regeneration that stem cells, mainly tissue-specific, may be involved in replacing damaged liver tissue. Theoretically, principles of hepatocyte transplantation can be also applicable with stem cell-derived hepatocyte-like cells, in terms of; cell numbers needed, site of application, mode of action, mechanisms of tissue integration and support of liver function for short or long periods.

1.6.1 ESCs

Human ESCs are usually derived from blastocyst stage embryos 5-6 day after *in vitro* fertilization. After their first discovery in 1998 (Thomson, Itskovitz-Eldor et al. 1998), a breakthrough in human ESC research has been initiated with the derivation of many cell lines and the improvement of cell culture conditions. Our laboratory actively participated in this field developing the standard non-fetal bovine serum (FBS)-containing culture system using human, rather than mouse feeders (Hovatta, Mikkola et al. 2003; Inzunza, Gertow et al. 2005) and introducing a new mechanical animal-reagent free, rather than enzymatic derivation technique (Strom, Inzunza et al. 2007). We have also developed a new feeder-free culturing system using human recombinant laminins (Rodin, Domogatskaya et al. 2010; Bergstrom, Strom et al. 2011).

Given the optimum culturing conditions, human ESCs have unlimited multiplication and differentiation potentials, making them an ideal candidate for regenerative medicine. Differentiation to hepatocytes has been successfully reported using mouse, monkey and human ESCs (Lavon and Benvenisty 2005). For mouse ESC induction to hepatocytes a hepatic induction factor cocktail (Teratani, Yamamoto et al. 2005) or co-culture with human non-parenchymal cells (Soto-Gutierrez, Navarro-Alvarez et al.

2007) have been used. For human ESC differentiation to hepatocytes, many differentiation protocols using activin A and HGF (Chen, Soto-Gutierrez et al. 2006), HGF and a synthetic fabric (Soto-Gutierrez, Navarro-Alvarez et al. 2006) have been used with varying degrees of success. Many other protocols trying to emulate the *in vivo* scenario of hepatocyte development have been published, first differentiating human ESCs to definitive endoderm then inducing hepatocyte differentiation followed by maturation induction (Basma, Soto-Gutierrez et al. 2009; Synnergren, Heins et al. 2010; Sivertsson, Synnergren et al. 2012).

1.6.2 iPSCs

In 2006, iPSCs were introduced for the first time as a trial to bypass both ethical and immunological rejection concerns regarding human ESC use in regenerative medicine (Takahashi and Yamanaka 2006). Direct reprogramming of human fibroblasts to assume a pluripotent state has been done by viral transduction using the transcription factors Oct4, Sox2, and either cMyc and Klf4 (Takahashi and Yamanaka 2006), or Nanog and Lin28 (Yu, Vodyanik et al. 2007). Extensive research is being carried out for more efficient reprogramming of iPSCs using safer techniques (Okita, Matsumura et al. 2011).

iPSCs have similar characteristics to those for ESCs in terms of; morphology, gene expression profiles, cell surface antigens and the high proliferative and differentiative capabilities (Ishikawa, Banas et al. 2012). Moreover, iPSCs are known to keep the same human leukocyte antigen (HLA) genotype of their parental somatic cells (Masaki, Ishikawa et al. 2007) giving the opportunity to treat patients using their own pluripotent stem cells. Similar to human ESCs, iPSCs showed their capability to differentiate to hepatocyte-like cells *in vitro* (Song, Cai et al. 2009; Si-Tayeb, Noto et al. 2010; Chen, Tseng et al. 2012), which then can be used in cell-based therapy for liver disease. Worthy of mentioning is that, iPSCs may also become immunogenic to their native host (Zhao, Zhang et al. 2011).

1.6.3 MSCs

MSCs were first identified in bone marrow (BM) as plastic-adherent fibroblast-like cells with colony-forming unit fibroblastic (CFU-F) properties (Friedenstein, Chailakhyan et al. 1974; Friedenstein, Latzinik et al. 1982; Friedenstein, Chailakhyan et al. 1987). An extensive research was then elicited, where many groups are trying to define, characterize, isolate and purify MSCs from different tissues. Yet, there is no clear cut definition for these cells with different nomenclature: mesenchymal stem cells, stromal stem cells, mesenchymal progenitors, among others. Relatively recently, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy suggested the name ‘multipotent mesenchymal stromal cells’ and put the minimal criteria for defining MSCs as: plastic adherence and CFU-F formation in standard culture conditions; with expression of CD73, CD90 and CD105; while lacking the expression of CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR surface markers (Dominici, Le Blanc et al. 2006). The ‘stemness’ of MSCs is debated as they still lack unique multipotency markers, compared to pluripotent stem cells. Cultured MSCs may lose some of their markers and acquire other non-specific markers, but surprisingly, they can still keep their multipotency (Jones and McGonagle 2008).

The possibility for MSCs to differentiate to hepatocyte-like cells were first suggested in 1999 (Petersen, Bowen et al. 1999), followed by another report few years later (Schwartz, Reyes et al. 2002). Many other reports came out supporting the same idea (Sato, Araki et al. 2005; Aurich, Sgodda et al. 2009), but with no strong evidence for MSC differentiation to hepatocyte-like cells having mature hepatocyte functions (Strom and Ellis 2011; Ishikawa, Banas et al. 2012).

MSCs can be obtained from patients, expanded *in vitro* and transplanted back as an autologous cell therapy. Moreover, MSCs are immunoprivileged with absent expression of HLA-II, making them ideal also for allogeneic cell therapy (Le Blanc, Tammik et al. 2003; Le Blanc, Frassoni et al. 2008). There is strong evidence for MSCs acting as 'trophic mediators' where they secrete bioactive reagents, which function as either immumosuppressors or promoters of regeneration (Caplan and Dennis 2006). Allogeneic MSC transplantation has been suggested to support hepatocyte survival, proliferation and function both *in vivo* and *in vitro* and to decrease the host allogeneic response to transplanted hepatocytes and modulate stellate cell activation in liver cirrhosis causing its regression (Isoda, Kojima et al. 2004; Gomez-Aristizabal, Keating et al. 2009). A promising phase-1 clinical trial strongly supported MSC clinical use in liver cirrhosis (Mohamadnejad, Alimoghaddam et al. 2007).

Fetal MSCs were suggested to have stronger therapeutic potentials being more primitive and having marked proliferative (Gotherstrom, West et al. 2005; Guillot, Gotherstrom et al. 2007) and differentiative (Chan, Waddington et al. 2007; Kennea, Waddington et al. 2009) capacities compared to their adult equivalents. Fetal MSCs were also able to inhibit lymphocyte proliferation induced by mitogens (Gotherstrom, Ringden et al. 2003).

1.7 CRYOPRESERVATION AND COLD STORAGE

1.7.1 Hepatocyte Cryopreservation

Hepatocyte cryopreservation is essential for the availability of hepatocytes and stem cell-derived hepatocyte-like cells for emergency as well as planned treatment for liver disease patients and also for providing hepatocytes for drug toxicity and metabolism studies. The first successful trials for hepatocyte cryopreservation were performed in rat in 1980 (Fuller, Morris et al. 1980) and in human in 1986 (Loretz, Li et al. 1989). Since then, many groups have made efforts in developing better cryopreservation protocols (Rijntjes, Moshage et al. 1986; Dou, de Sousa et al. 1992; Li, Lu et al. 1999; Alexandre, Viollon-Abadie et al. 2002; Hewitt 2010; Terry, Dhawan et al. 2010). In spite of such efforts, significant loss of viability and function after thawing is still a major problem calling for further optimization for available hepatocyte cryopreservation protocols.

The key factors in hepatocyte cryopreservation are: FBS, DMSO and the university of Wisconsin solution (UW). FBS supports hepatocytes during cryopreservation by: containing several growth and attachment factors, metal transporters, protease inhibitors, among others; providing mechanical protection for cells; and having a buffering capacity. However, it is not suitable for clinical purposes because of its animal origin. UW, on the other hand, is a xeno-free solution that helps support hepatocytes during cryopreservation by: minimizing injury from hypothermia and oxygen free radicals, preventing intracellular acidosis and interstitial tissue expansion as well as providing a source of energy for hepatocytes. DMSO, the standard

cryoprotectant allows for only a moderate increase in solute concentration during freezing and preserves membrane integrity by interacting with membrane phospholipids (Hewitt 2010).

1.7.2 Hepatocyte Cold Storage

During hepatocyte transplantation, it has been shown that only what equals to 5% of the total liver mass can be infused at a time. Hepatocyte infusion has to be performed slowly over several hours to days to avoid the risk for portal vein thrombosis (Fox, Chowdhury et al. 1998). This necessitates the need for an efficient cold preservation method for hepatocytes.

Cold storage of hepatocytes aims at storing them for shorter periods without cryopreservation. A hepatocyte preservation solution is needed and many solutions such as UW, Histidine-tryptophan-ketoglutarate (HTK) and Celsior have been tested (Abrahamse, van Runnard Heimel et al. 2003). Several studies have succeeded in cold storage of isolated hepatocytes for more than 3 days, albeit with marked loss of viability and function (Gramignoli, Marongiu et al. 2011; Pless, Sauer et al. 2012).

2 AIMS

The main aim for this thesis was to study various aspects of cellular therapy for liver disease as an alternative to liver transplantation. To fulfill this aim, 4 studies were carried out with the following specific aims:

1. To identify and characterize a mesenchymal stem cell population within the human fetal liver, and evaluate the cells' differentiation potential to hepatocytes.

2. To profile and identify microRNAs important for the development in human fetal and adult liver.

3. To optimize a xeno-free cryopreservation protocol for primary human hepatocytes.

4. To optimize a cold storage protocol for primary human hepatocytes.

3 MATERIAL AND METHODS

3.1 ETHICS STATEMENT

Human fetal liver samples were used for MSC isolation and immunohistochemistry (paper I & preliminary data) and for miRNA isolation (paper II). The samples were obtained from fetuses aborted during the first trimester by modified vacuum curettage from volunteered women who donated the fetal tissues. An informed written consent was obtained from each donor.
Human adult liver samples were used for obtaining primary hepatocytes (papers II, III, & IV). The samples were also used for immunohistochemistry (paper I) and TUNEL staining (paper IV). Used samples were obtained from liver resections or from deceased organ donors. An informed written consent was obtained for each sample.
Peripheral blood samples were taken from healthy volunteers who have donated the blood (paper I). An informed written consent was obtained from each donor.
C57 black/nude mice (preliminary data) were housed at the animal facility, Karolinska University Hospital, Huddinge, Sweden. The animals were housed according to the Animal Care and Use Facility at the Karolinska University Hospital.
All studies were approved by the Regional Ethical Review Board in Stockholm, Sweden and the IRB protocol, University of Pittsburgh, PA, USA. The studies were done in compliance with the international guidelines regarding the use of human tissue in research.

3.2 CELL ISOLATION

3.2.1 MSCs from Human Fetal Liver

To isolate MSCs from human fetal liver (hFL-MSCs), single cell suspension was first prepared (paper I). Fetal liver samples were gently disintegrated by pressing them against a 70μm cell strainer. CD271, an MSC marker, was used to positively isolate a pure population of MSCs by adding magnetic beads labeled with CD271 to the cell suspension. After isolation, hFL-MSCs in the positive fraction were cultured in MSC medium, composed of Dulbecco's Modified Eagle's Medium Low Glucose (DMEM-LG), supplemented with 10% FBS, 1% Glutamax and 1% penicillin-streptomycin.

3.2.2 Primary Human Hepatocytes

Hepatocytes were isolated using a three-step collagenase perfusion procedure (papers II, III & IV). First, liver samples were perfused with warm (37°C) Hank's buffered salt solution (HBSS) containing ethylene glycol tetraacetic acid (EGTA) to flush blood out of the sample. As a calcium chelating agent, EGTA helps disrupt cell adhesion to extracellular matrix. Second, perfusion with warm HBSS alone was done to wash out remaining EGTA, as digestive enzymes are dependent on calcium and magnesium for their activation. Finally, liver tissue was perfused with warm Eagle's Minimum Essential Medium with Earle's salts containing collagenase XI for enzymatic dissolution. Undigested tissue pieces were removed by filtration through sterile gauze while hepatocytes were collected by low speed centrifugation. Hepatocyte medium was composed of Williams E medium without phenol red supplemented with 5% FBS, insulin (10^{-7}M), dexamethasone (10^{-7}M) and an antibiotic-antimycotic.

3.3 ASSESSMENT OF CELLULAR VIABILITY

The trypan blue exclusion method (TB) was used for assessment of cell viability indirectly by evaluating cell membrane integrity (papers I, III, & IV). Dead cells with disrupted cell membrane allow the passage of trypan blue through the membrane, which in turn stains the cytoplasm blue.
In (paper III), in addition to TB, viability of the hepatocytes was further evaluated by the Live/Dead Assay (LDA). LDA is a two-colour viability assay where two fluorescent probes are used for assessing cell membrane integrity and intracellular esterase activity; ethidium homodimer-1 (EthD-1) and calcein acetoxymethyl (calcein AM), respectively. Live cells actively take up the non-fluorescent cell-permeable calcein AM where intracellular esterase cleaves it producing the intensely fluorescent calcein. EthD-1 crosses damaged cell membrane and binds nucleic acids.

3.3.1 Apoptosis Assays

The previous methods for viability assessment mainly detect metabolically non-viable necrotic cells, which have lost their membrane integrity. Apoptosis assays aim at detecting the early events in programmed cell death (paper IV). Apoptotic cells are metabolically viable cells having; a blebbing, albeit intact, cell membrane, uniformly condensed chromatin, uniformly fragmented DNA and with specific enzyme cascades initiated (Thompson, Strange et al. 1992).

3.3.1.1 TUNEL Test

The TUNEL test is designed for the detection of uniform nucleosome-sized DNA fragments by labeling their 3'OH termini *in situ* with modified nucleotides. The nucleotides are fixed to the DNA by the enzyme terminal deoxynucleotidyl transferase (TdT). TdT labels both single-stranded and double-stranded DNA fragments (Gavrieli, Sherman et al. 1992). Anti-digoxigenin antibody labeled with a peroxidase reporter molecule binds digoxigenin-conjugated nucleotides. DAB, a peroxidase chromogenic substrate, is added to the treated liver tissue and cytospinned hepatocyte slides for color development. Positive cells were counted under light microscopy.

3.3.1.2 Caspase 3/7 Assay

Caspases are a family of proteases playing key roles in apoptosis in mammalian cells. Caspases 3 and 7 are important members in this family (Bayascas, Yuste et al. 2002). Upon treating lysed hepatocytes with a luciferin-derived caspase substrate, caspases cleave it freeing luciferin and generating a luminescent signal to be detected by a luminometer. The luminescence generated is proportional to caspase activity reflecting the degree of apoptosis within hepatocytes.

3.4 CHARACTERIZATION OF HUMAN FL-MSCS

3.4.1 Marker Expression

hFL-MSCs were characterized on both the protein and functional levels. The expression of known mesenchymal and lack of expression of non-mesenchymal markers were demonstrated by flow cytometry analysis and immunocytochemical

staining (ICC) (paper I). The markers tested by flow cytometry included CD271, CD31, CD73, CD90, CD80, CD86, CD105, CD44, CD14, CD34, CD45, HLA-DR, HLA class I and HLA class II. Mesenchymal marker expression was further confirmed by ICC for CD271, CD90, CD34, CD45, CD73, CD105 and CD166.
Being derived from human fetal liver, hFL-MSCs were also examined for their expression of certain hepatic markers. ICC staining was done for α-fetoprotein (AFP), albumin (ALB), CK18 and CK19 (paper I).

3.4.2 Differentiation Potential

A mesenchymal origin for hFL-MSCs was further confirmed by evaluating their differentiation potential towards adipogenic and osteogenic lineages *in vitro* (paper I). The potentiality for hFL-MSCs to differentiate towards hepatogenic lineages was also tested *in vitro* (paper I).

3.4.2.1 Osteogenic Differentiation

For osteogenic stimulation (paper I), hFL-MSCs were treated with DMEM-LG supplemented with dexamethasone, glycerophosphate, ascorbic acid-2-phosphate, FBS and penicillin-streptomycin. Von Kossa staining, based on the use of silver nitrate, detects calcium phosphate deposited in the extracellular matrix by differentiating hFL-MSCs. Calcium is reduced by light exposure and gets replaced by silver ions forming silver phosphate, which is then degraded by light forming silver. Alizarin red S directly binds calcium in the extracellular matrix by labelling its deposits in differentiating hFL-MSCs.

3.4.2.2 Adipogenic Differentiation

For adipogenic stimulation (paper I), hFL-MSCs were alternatively treated with an induction medium composed of: DMEM-high glucose (DMEM-HG), 1-methyl-3-isobutylxanthine, insulin, indomethacin, dexamethasone, FBS as well as penicillin-streptomycin and a supportive medium composed of: DMEM-HG, insulin, FBS and penicillin-streptomycin. Fat droplets within differentiating hFL-MSCs were detected by Oil Red O staining, where Oil Red directly labels lipids.

3.4.2.3 Hepatogenic Differentiation in vitro

Whether hFL-MSCs were able differentiate to hepatocytes *in vitro* (paper I) was determined by treating them with an induction medium containing: Iscove's modified Dulbecco's medium (IMDM) with HGF, basic FGF, nicotinamide and insulin-transferrin-selenium (ITS) and thereafter a maturation medium containing: IMDM with oncostatin M, dexamethasone and ITS. hFL-MSCs were stained for hepatic marker expression and tested for CYPs activity (see below).

3.4.2.4 Hepatogenic Differentiation in vivo (Preliminary)

hFL-MSC potentiality to differentiate to hepatocytes *in vivo* was preliminary tested by their transplantation to C57 black/nude mice. hFL-MSCs isolated using a cell depletion cocktail including monoclonal antibodies to the following lineage specific cell surface antigens (Glycophorin A, CD3, CD14, CD19, CD66b and CD38) were used. hFL-MSCs were transduced with green fluorescent protein (GFP). GFP transduction was

done using a recombinant lentiviral vector produced using a 3-plasmid expression system as described elsewhere (He, Ehrnfelt et al. 2004). One million cells were injected in a volume of 300µl DMEM-LG intra-splenic after partial hepatectomy to give a space advantage for the transplanted cells. Mice were injected with retrorsine, 70mg/kg, intra-peritoneal a week before transplantation. Retrorsine inhibits host hepatocyte proliferation giving, in addition, a growth advantage for the transplanted hFL-MSCs. In addition to five hFL-MSC-transplanted animals, two animals were sham-operated and two more were kept as control. Fresh frozen cryosections of mouse liver were examined for GFP expression and ICC-stained for the following human hepatic markers; ALB, AFP, CK18 and CK19.

3.4.3 Immunomodulatory Effects

To test for immunogenicity and immunomodulatory properties of hFL-MSCs (paper I), mixed lymphocyte cultures (MLCs) were performed with adult peripheral blood lymphocytes (PBLs). Triplicate samples of PBLs were cultured with irradiated autologous or allogeneic PBLs pooled from five donors with or without irradiated hFL-MSCs. The immunogenicity and immunomodulatory properties of hFL-MSCs were similarly tested in MLCs including hFL-MSCs, PBLs and HepG2 cells, a human hepatocellular carcinoma cell line. The immunomodulatory aspects of hFL-MSCs are of interest for their potential transplantation to patients with liver disease, either alone or together with other cell types, such as hepatocytes.

3.5 MICRORNA PROFILING

Stem cell differentiation to hepatocytes is extensively studied, and huge effort is being employed for optimization of differentiation protocols. Our attempts to differentiate hFL-MSCs to hepatocytes both *in vitro* and *in vivo* are mentioned in this thesis. A potential role for miRNAs in liver development has been widely suggested and a miRNA-based stem cell differentiation protocol is a future aim. Extensive microarray profiling for miRNA expression in human fetal and adult liver has been initiated here (paper II). The aim was to identify the key miRNA players in liver development.

3.5.1 Microarray Analysis

Total RNA was extracted from fetal and adult liver samples using a miRNA-specific extraction kit to preserve small RNA fraction during extraction. Total RNA concentration, quality and integrity were analyzed prior to microarray hybridization. Microarray analysis was performed to compare the expression of individual miRNAs in the different liver samples. Microarray data were processed with the R software package where linear models followed by moderated t-test analyses were used to identify differentially expressed miRNAs between adult and fetal liver samples.

3.5.2 miRNA Target Prediction

Target prediction aimed at identifying potential gene targets for enriched miRNAs. miRNAs with at least two-fold higher expression were selected for target prediction bioinformatically using the miRWalk Database. Similarity scores were calculated for the predicted targets upon comparing them to previously published lists of specific tissue selective genes. Genes intersected between predicted and listed fetal liver genes

were identified as candidate gene set for fetal liver miRNAs. Another candidate gene set for adult liver miRNAs was similarly prepared.

3.5.3 Pathway Analysis

Pathway analysis aimed at the bioinformatic linking of specific miRNA target genes to specific physiological/pathological processes. Both candidate gene lists prepared before for fetal and adult liver miRNAs were tested. Results from target pathway analysis were further discussed against previously published data.

3.5.4 Quantitative RT-PCR Analysis (qRT-PCR)

qRT-PCR analysis aimed at confirming the microarray data. The three highly expressed miRNAs in the fetal liver samples and the two highly expressed miRNAs in the adult liver samples were selected for further qRT-PCR confirmation in three new fetal and three new adult liver samples. miRNA relative expression in the adult and fetal samples was calculated and t-test values were estimated.

3.6 CHARACTERIZATION OF HEPATOCYTES

3.6.1 CYPs Activity

Primary human hepatocytes and hFL-MSCs differentiated to hepatocytes were tested for their CYPs activity (papers I, III, & IV). Activity of the major CYPs; CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A7 was assessed. Luminescence-based assays utilizing luciferin-derived P450-Glo substrates and their specific luciferin detection reagents were used. CYPs inside the cells metabolize substrates and free luciferin, which is then metabolized by luciferase in the detection reagent. Activity of the CYPs was estimated as the degree of luminescence emitted from hepatocytes. CYPs were sometimes induced by β-naphthoflavone (BNF) (CYP1A1/2), rifampicin (RIF) phenobarbital (PB) (CYP 3A4) or DMSO (vehicle control) (paper IV).

3.6.2 7-Ethoxyresorufin Metabolism

Activity of CYP1A1/2 was also tested by the ability of the cells to convert 7-ethoxyresorufin to resorufin (paper IV). The conversion is effected by the 7-ethoxyresorufin-O-deethylase (EROD) enzyme (Burke and Mayer 1974). Cells were incubated with 7-ethoxyresorufin, and the fluorescence of the outproduct resorufin reflected CYP1A1/2 activity. Salicylamide was added to block the phase II enzymes metabolizing resorufin itself. A resorufin sodium standard curve was run in parallel to quantify the amount of resorufin formed.

3.6.3 Resorufin Metabolism

While CYPs activity reflects phase I liver activity, resorufin metabolism reflects phase II function. Resorufin undergoes glucuronidation and sulfation by phase II enzymes forming non-fluorescent compounds (Gonzalez, Reimschuessel et al. 2009). Cells were incubated with resorufin and the decrease in its fluorescence was measured (paper IV). Similarly, a resorufin sodium standard curve was run in parallel to quantify the amount of resorufin loss.

3.6.4 Ammonia Detoxification

Ammonia detoxification is a major hepatic function, and hepatic encephalopathy due to ammonia accumulation is a serious problem accompanying liver cell failure. Five enzymatic reactions are involved in the urea cycle of converting ammonia into urea, which is then eliminated through the kidneys.
Cells were incubated with ammonium chloride and three reagents were used for colorimetric estimation of ammonia clearance (paper IV). Reagent A contained sodium tungstate, as well as sulphuric and phosphoric acids; reagent B contained phenol and sodium nitroprusside; while reagent C had potassium carbonate and sodium hypochlorite. Ammonia reacts with hypochlorite forming monochloramine that reacts with phenol forming an indophenol derivative with a deep blue color (Brautigam, Gagneul et al. 2007). An ammonium chloride standard curve was run in parallel to quantify ammonia metabolism.

3.6.5 Plating Efficiency

Plating efficiency is the ability of hepatocytes to attach to the culture dish and is considered as an indicator of hepatocyte ability to engraft into the host liver after transplantation (Wan, Zhang et al. 2012). Plating efficiency was calculated as the protein content of attaching hepatocytes divided by that of both attaching and non-attaching hepatocytes (paper IV). A protein harvesting solution containing; Tris, potassium chloride, EDTA and protease inhibitor was used for collecting protein. A colorimetric protein assay based on the Coomassie blue dye was used for protein estimation. Coomassie blue binds to primarily basic and aromatic amino acid residues, especially arginine (Compton and Jones 1985). A protein standard curve, e.g. using bovine serum albumin (BSA), was run in parallel for quantification.

3.6.6 Normalization of Enzyme Activity

Results of the functional assays were related to the actual number of the cells tested. This was done by normalizing the results to one million viable cells, or to the amount of protein or DNA within the cells tested. Protein content was estimated as described before for plating efficiency calculation. A Hoechst 33258-based fluorescent assay selective for double stranded DNA (dsDNA) was used. A dsDNA standard curve was run in parallel for quantification.

3.7 COLD PRESERVATION

Saving cells for longer periods, through cryopreservation, or for shorter periods, through cold storage, is an essential component of clinical cell transplantation in liver disease. This applies to primary human hepatocytes, stem cell-derived hepatocyte-like cells and any other candidate cell type. Primary human hepatocytes, the first candidate for transplantation, were used for optimizing both cryopreservation and cold storage protocols.

3.7.1 Cryopreservation of Hepatocytes

It has been suggested that controlled rate freezing (CRF) in special freezers is superior to ordinary polystyrene box freezing (PSB). A comparison between both methods was carried out (paper III).

3.7.1.1 Controlled Rate Freezing

Hepatocytes in (UW + 12% DMSO) were transferred to cryotubes on ice. Half of the tubes were frozen in a controlled rate freezer and transferred to the vapor phase of a liquid nitrogen tank. The other half of the tubes were wrapped in tissue paper and put into a common laboratory PSB. The box was sealed, shut with a tape and quickly placed into a -70°C freezer. After two days, the frozen tubes were transferred to the vapor phase of a liquid nitrogen tank for storage.

3.7.1.2 Polystyrene Box Freezing

Two different cryopreservation solutions have been tested, the standard (UW + 12% DMSO) and a new xeno-free medium, containing 10% DMSO (permeating) and a high polymer anhydrous dextrose (non-permeating) cryoprotectants. The cryotubes were placed in PSB and kept at -70°C for overnight and then moved to the vapor phase of a liquid nitrogen tank for storage. When thawed, cryotubes were first incubated in a water bath at 37°C for 1-2 minutes, and then cells were resuspended in cold hepatocyte medium. For cells frozen in the new solution, a specific thawing buffer was used before the cells being resuspended in the hepatocyte medium.
Viability of the hepatocytes using trypan blue and LDA was compared between fresh hepatocytes, the two box freezing protocols and the CRF method. CYPs activity was also compared among the different groups.

3.7.2 Cold Storage of Hepatocytes

For cold storage, preservation of whole liver tissue was compared to preservation of single hepatocytes (paper IV). UW was used in both protocols. Liver tissue was divided into two pieces and hepatocytes were isolated as mentioned above. Hepatocytes were analyzed directly after isolation (fresh) or after storage for 48 hours at 4°C in UW, referred to as (UW cells). Liver tissue from the same donor was stored at 4°C in UW and hepatocytes were isolated after 48 hours, referred to as (UW tissue cells). Viability of the hepatocytes was evaluated in both groups as mentioned above. Hepatocyte plating efficiency, ammonia metabolism, activity of CYP1A1/2, -2C9, -3A7 and -3A4, phase II conjugation as well as apoptosis evaluation by TUNEL assay and caspase 3/7 activities were also compared between groups as described above.

4 RESULTS

4.1 MSCS IDENTIFIED IN HUMAN FETAL LIVER

Data in (paper I) suggested the presence of an MSC population in human fetal liver, which we named hFL-MSCs. hFL-MSCs that were positively isolated using CD271-labelled magnetic beads were a homogenous population.

4.1.1 CD271 positive Cells in Human Liver

Cultured fetal liver cells as well as fetal and adult liver sections showed positive staining for the mesenchymal marker CD271 (figure 4.1). CD271 expression was higher in the fetal liver (70.1%) than in the adult (23.4%).

4.1.2 Mesenchymal Phenotype

hFL-MSCs had a mesenchymal immunophenotype upon ICC staining and flow cytometry analysis. By flow cytometry, cultured hFL-MSCs were positive for; CD105 (SH2), CD44, CD73 (SH4), CD90 (Thy1) and HLA class I (more than 90%) and negative for; CD14, CD45, CD31, CD34, CD80, CD86, HLA class II and HLA-DR (less than 5%). hFL-MSCs were partially positive for CD271 (17-20%).

4.1.3 Mesodermal Differentiation

hFL-MSCs showed the ability to differentiate towards osteogenic and adipogenic lineages *in vitro* as shown by Von Kossa/Alizarin Red staining and Oil Red O staining, respectively (figure 4.2). Staining quantification showed that different samples had different differentiation potentiality.

4.1.4 Hepatogenic Differentiation

Despite their liver origin and their prior expression of hepatic markers, hFL-MSCs did not differentiate, in our hands, towards hepatic lineages when stimulated *in vitro*. Differentiating cells did not show any CYPs activity and even started to lose their hepatic marker expression as noticed from ICC staining.

4.1.5 Immunomodulatory Properties

MLCs suggested that hFL-MSCs were non-immunogenic when 10% hFL-MSCs were co-cultured with peripheral blood lymphocytes (PBLs) *in vitro*, as they did not elicit an immune response (n=6) (figure 4.3).
When cultured with PBLs and HepG2 cells, hFL-MSCs continued to be non-immunogenic and even modulated the PBL immune response to the HepG2 cells. When 10% hFL-MSCs was added to co-cultures of PBLs and HepG2 cells, hFL-MSCs had variable effects on allogeneic PBLs responses towards HepG2 cells: one hFL-MSC sample markedly inhibited lymphocyte proliferation by 63.3% (STDEV 37.2%) the second sample did not have any marked effect on lymphocyte proliferation and the third sample even stimulated lymphocyte proliferation by 22% (STDEV 24.8%) compared to MLC with HepG2 (n=6) (figure 4.3).

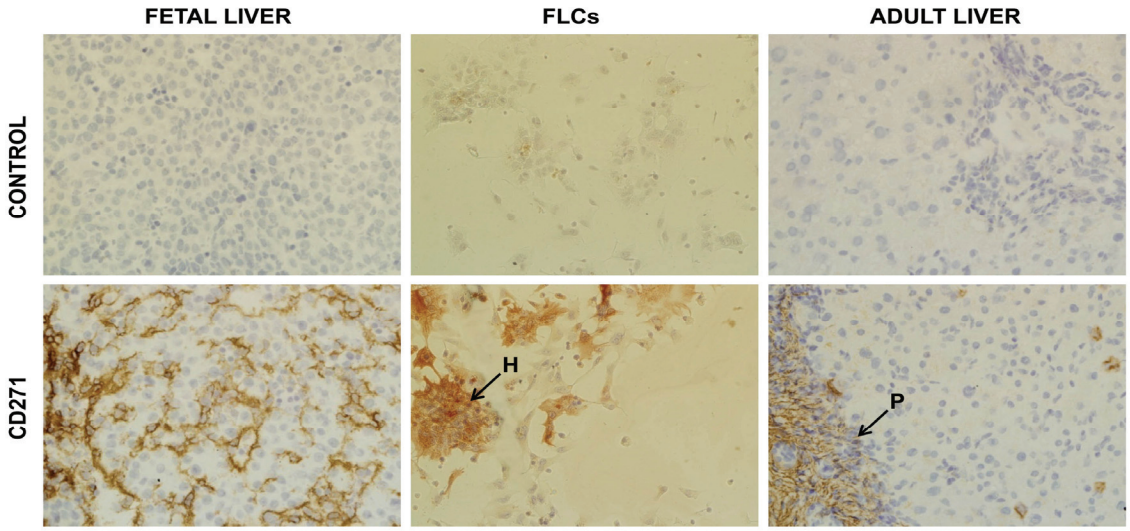

Figure 4.1: Enzymatic peroxidase immunochemical staining for CD271 expression in frozen sections from human fetal and adult liver as well as fetal liver cells (FLCs). Isotype controls were included. DAB (in fetal and adult liver sections) and AEC (in FLCs) substrates were used for color development. Brown color indicates positive staining. Some hepatoblasts (H) stained positive for CD271. In the adult liver, CD271 was mainly expressed in the portal area (P). Magnification 20X.

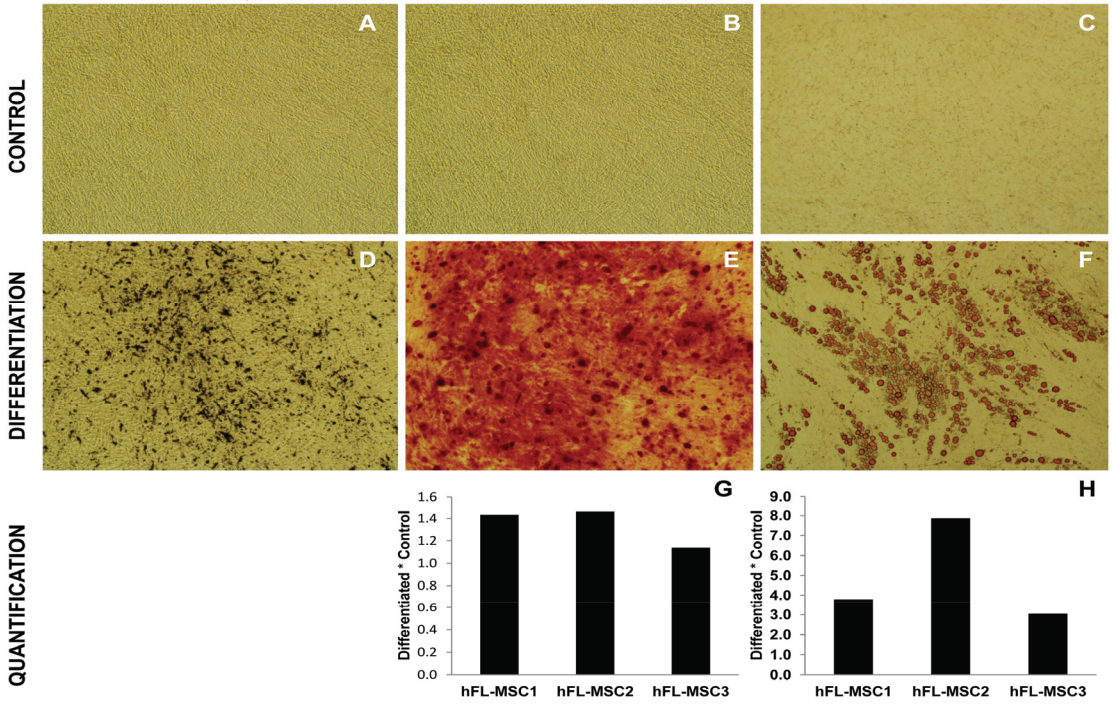

Figure 4.2: Osteogenic and adipogenic differentiation of CD271-isolated mesenchymal stem cells from human fetal liver (hFL-MSCs). hFL-MSCs from passages 3-5 were stimulated with osteogenic (3 weeks) or adipogenic (3 cycles) media. An aliquot of cells were grown in MSC medium as a control. Control (A, B) and osteogenic stimulated hFL-MSCs were stained with Von Kossa (D, black staining) and Alizarin red S (E, red staining) for calcium deposition. Control (C) and adipogenic stimulated (F) hFL-MSCs were stained with Oil red O to visualize lipid droplets (red staining). Typical pictures are shown for the differentiation. Magnification 20X. Alizarin red S staining of 3 osteogenic stimulated hFL-MSC samples (hFL-MSC1-3) was eluted by addition of 10% (w/v) cetylpyridinium chloride (G). Oil red O staining of 3 adipogenic stimulated hFL-MSC samples (hFL-MSC1-3) was eluted by addition of 100% isopropanol (H). Absorbance was measured at 500 nm (Oil red O) or at 562 nm (Alizarin red S) and plotted as differentiated hFL-MSCs times control hFL-MSCs. All samples were performed in duplicates.

4.1.6 hFL-MSC Transplantation to Mice (Preliminary Data)

MSCs previously isolated from human fetal liver and transduced with GFP were transplanted into mice after partial hepatectomy. The preliminary results suggested successful homing, engraftment, and survival of transplanted hFL-MSCs for at least two weeks after transplantation. Fresh frozen cryosections from transplanted mice livers clearly showed the green fluorescence of GFP-transduced cells. In addition, ICC staining of mouse liver sections for human specific ALB, AFP, CK18 and CK19 suggested the presence of human hepatocyte-like cells within the liver in the hFL-MSC-transplanted group (figure 4.4).

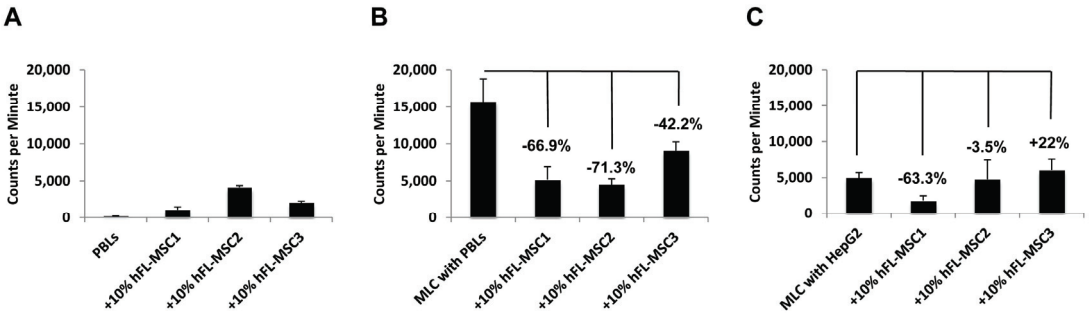

Figure 4.3: Mixed lymphocyte cultures (MLCs) with mesenchymal stem cells from human fetal liver (hFL-MSCs). 10% irradiated third party hFL-MSCs were co-cultured for six days with 200,000 responder and/or stimulator cells. Counts-per-minute depicts thymidine incorporation in the responder cell DNA. Mean ± STD of 6 experiments. **(A)** Lymphocyte immunological reaction towards hFL-MSCs at 6 days. **(B)** hFL-MSCs were co-cultured for six days with responder peripheral blood lymphocytes (PBLs) and an irradiated pool of stimulator PBLs, respectively. **(C)** hFL-MSCs were co-cultured for six days with responder PBLs and irradiated stimulator HepG2 cells, respectively.

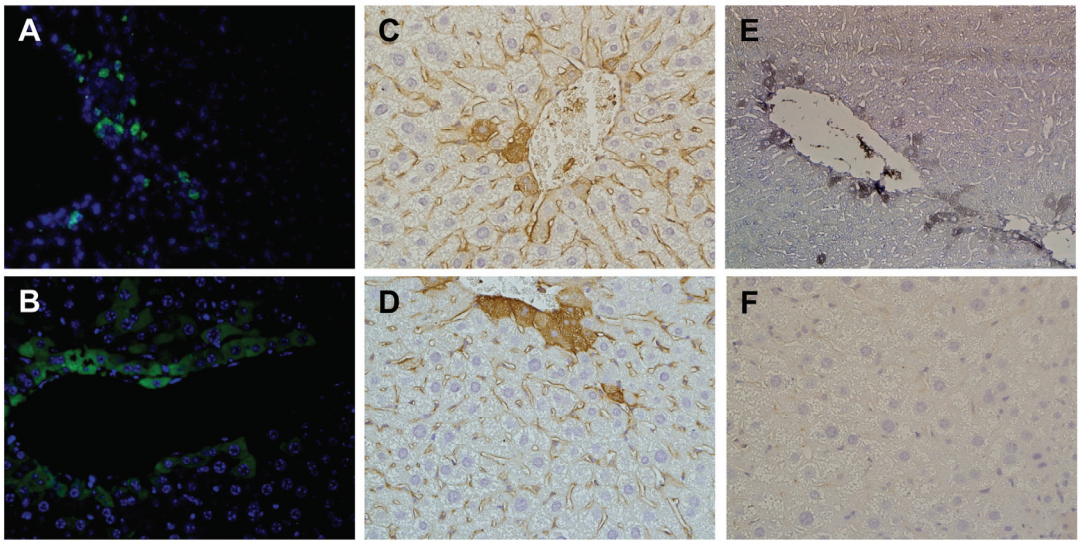

Figure 4.4: Liver sections from mice transplanted with hFL-MSCs: Fresh frozen section showing GFP-transduced hFLMSCs (A). Paraffin section stained with a FITC-conjugated anti-human albumin antibody (B). Peroxidase staining for anti-human antibodies for α-fetoprotein (C), CK19 (D), CK18 (E) and their isotype control (F) and substrates used for colour development were DAB (C,D) and DAB-Ni (E).

4.2 MICRORNAS PROFILED IN HUMAN LIVER

Extensive profiling for miRNA expression in human fetal and adult livers was done by microarray analysis (paper II). Bioinformatic target prediction and pathway analysis suggested the involvement of enriched miRNAs in fetal liver development and erythropoiesis.

4.2.1 Microarray Analysis

Microarray data identified 154 miRNAs to be significantly expressed in both fetal and adult liver samples. Of these, 74 miRNAs were expressed on a higher level in the fetal compared to the adult liver, with 48 miRNAs having at least two-fold higher expression. Similarly, 80 miRNAs were expressed on a higher level in the adult compared to the fetal liver, with 37 miRNAs having at least two-fold higher expression. Many of the listed miRNAs have been previously linked to fetal liver development. In addition, many of the highly expressed fetal miRNAs were previously linked to erythropoiesis.

4.2.2 Target Prediction

miRWalk database analysis linked 24 out of the 48 fetal miRNAs and 7 out of the 37 adult miRNAs to 12588 and 8474 gene targets respectively. Identified targets were compared to previously published target lists of tissue specific miRNAs (figure 4.5). Similarity testing between predicted and previously published targets identified a candidate gene set of 60 targets for fetal miRNAs (fetalGS) and of 26 targets for adult miRNAs (adultGS) to be cross-listed.

4.2.3 Pathway Analysis

Further pathway analysis for both gene sets linked selected miRNAs to certain physiological processes, such as extracellular matrix-receptor integration, complement and coagulation cascade, steroid hormone biosynthesis, glycolysis and gluconeogenesis (figure 4.6a&b). Many of these processes are known to be related to liver development and/or function.

4.2.4 Quantitative RT-PCR Confirmation

The qRT-PCR results confirmed the microarray data for selected miRNAs. Differentially expressed fetal and adult miRNAs continued to have similar expression pattern in the qRT-PCR.

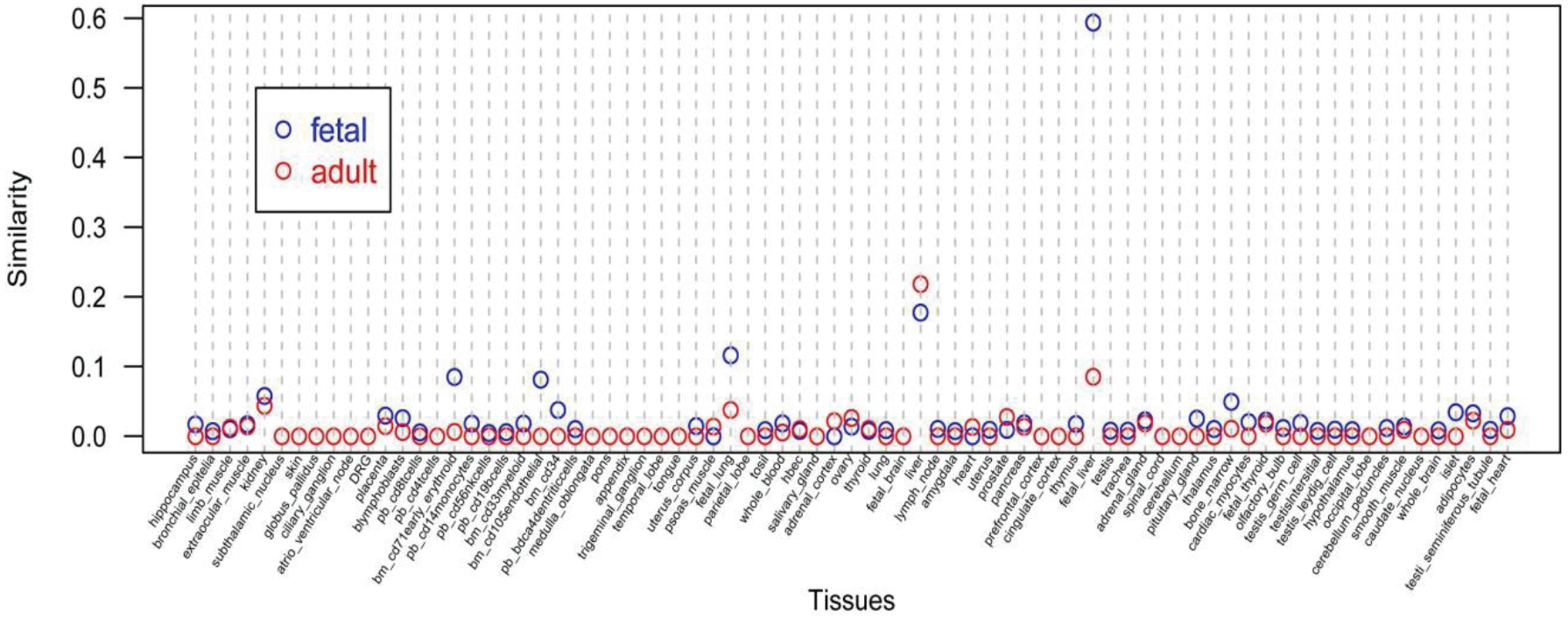

Figure 4.5: Comparison between targets for selected miRNAs and tissue-selective gene sets published by Greco et al., 2008 (Greco, Somervuo et al. 2008). Similarity values between fetal enriched and published targets are presented with blue circles and those for adult targets are shown in red. Similarity between predicted and published lists was higher in case of fetal and adult liver tissues.

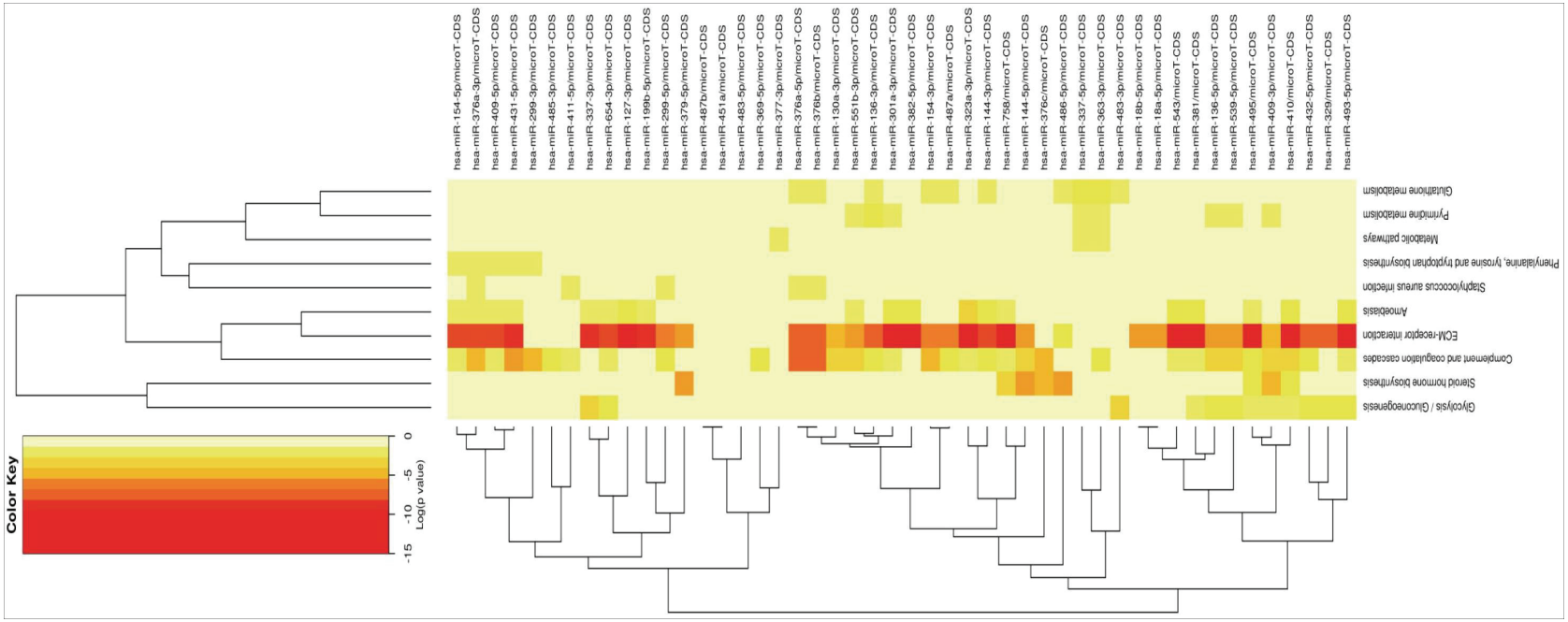

Figure 4.6a: Pathway analysis for the target set for selected fetal liver miRNAs. The dentogram shows miRNAs and the pathways linked to them. Darker color indicates stronger correlation. Selected fetal liver miRNAs were obviously linked to extracellular matrix-receptor integration, glucose metabolism, coagulation cascades and steroid hormone biosynthesis.

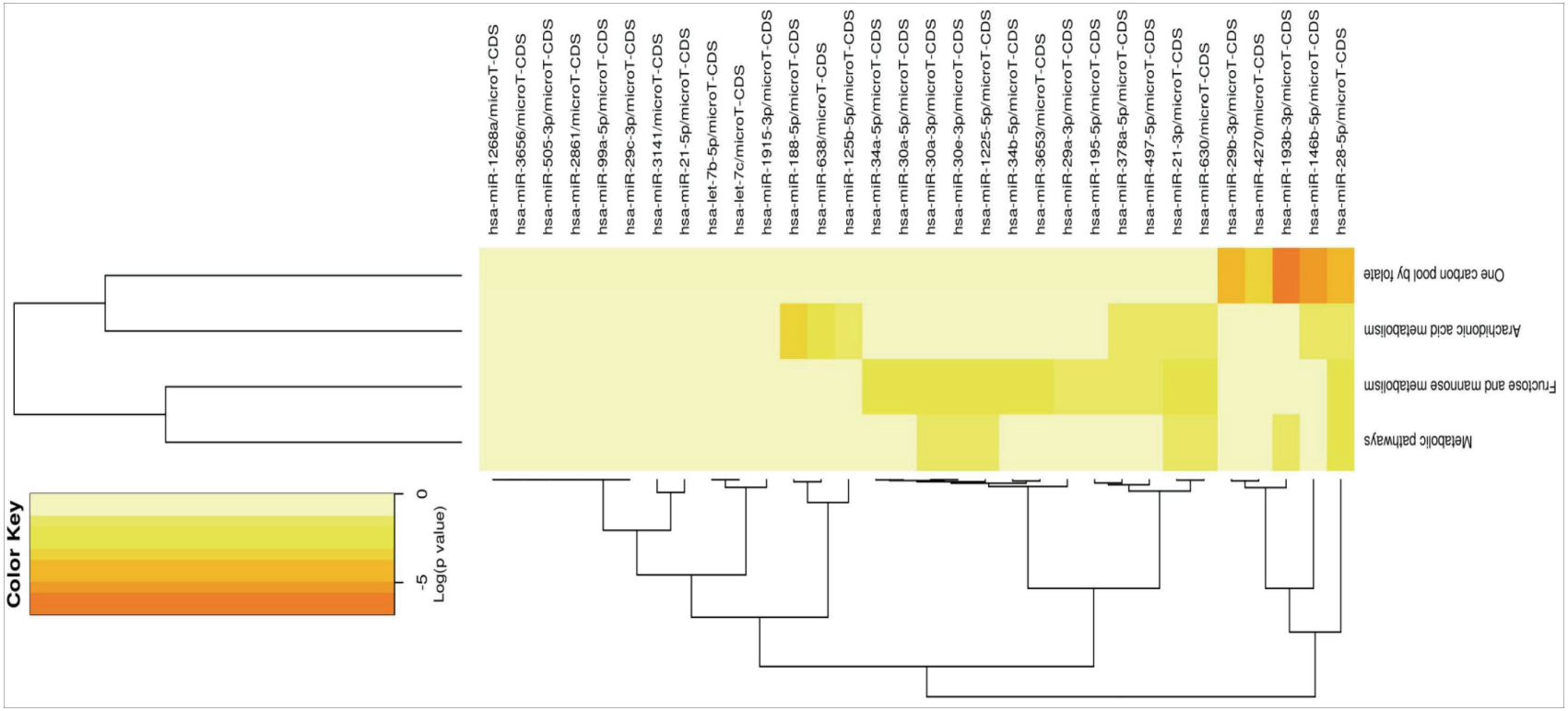

Figure 4.6b: Pathway analysis for the target set for selected adult liver miRNAs. The dentogram shows miRNAs and the pathways linked to them. Darker color indicates stronger correlation. Selected adult liver miRNAs were obviously linked to metabolic functions such as metabolism of fructose, mannose and arachidonic acid.

4.3 NEW XENO-FREE HEPATOCYTE FREEZING PROTOCOL

Two important comparisons, CRF to ordinary box freezing and two box freezing protocols were done in (Paper III).

4.3.1 Controlled-rate Freezing

In our hands, CRF did not appear to be superior to, but even worse than ordinary PSB freezing as noticed from hepatocyte viability (table 4.1).

Viability	Fresh%	PSB%	CRF%
L1	75	22	6
L2	74	38	22
L3	83	42	18
L4	73	40	37

Table 4.1: Comparison between controlled rate freezing (CRF) and polystyrene box freezing (PSB). PSB gave a viability of (35.5 ± 9.2), not significantly different from the freshly isolated (76.3 ± 4.6). CRF gave a significantly lower viability (2.8 ± 12.8) ($p<0.01$) compared to fresh. Figures represent percent ± SD of viable cells using trypan blue.

4.3.2 New PSB Freezing Protocol

In paper III, we introduced a new xeno-free hepatocyte cryopreservation protocol, referred to as (CB). The CB protocol seemed to be superior to the standard protocol, referred to as (DMSO-UW). Cell viability was compared between freshly isolated and cryopreserved hepatocytes using TB and LDA. Viability using CB was significantly higher than that using DMSO-UW (figure 4.7).

4.3.3 CYPs Activity

Although there was a tendency for CYPs activity to be a bit higher in the CB compared to DMSO-UW group, high within-groups variability seemed to preclude any between-group differences. This was manifested by the high standard deviation values that exceeded mean values in many cases (figure 4.8).

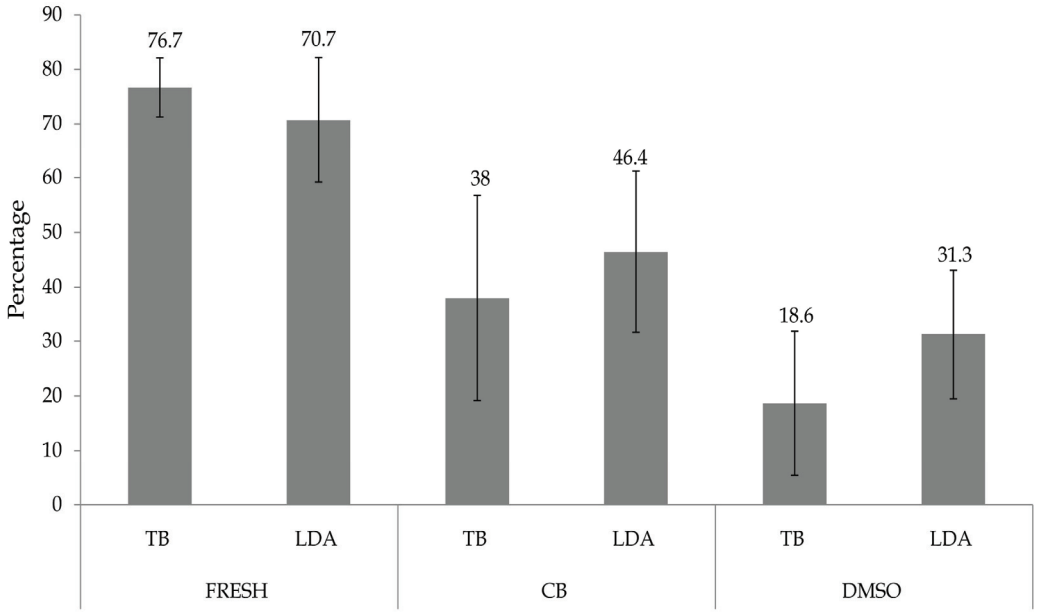

Figure 4.7: Hepatocyte viability before and after freezing: Viability in the three groups; fresh hepatocytes (FRESH) and hepatocytes frozen using either the new protocol (CB) or the standard DMSO-UW protocol (DMSO) using both the trypan blue exclusion and the Live/Dead Assay (LDA) methods. There was a significant difference in viability between CB and DMSO using the two-way ANOVA test ($P < 0.05$). Figures represent percent ± SD of viable cells using TB.

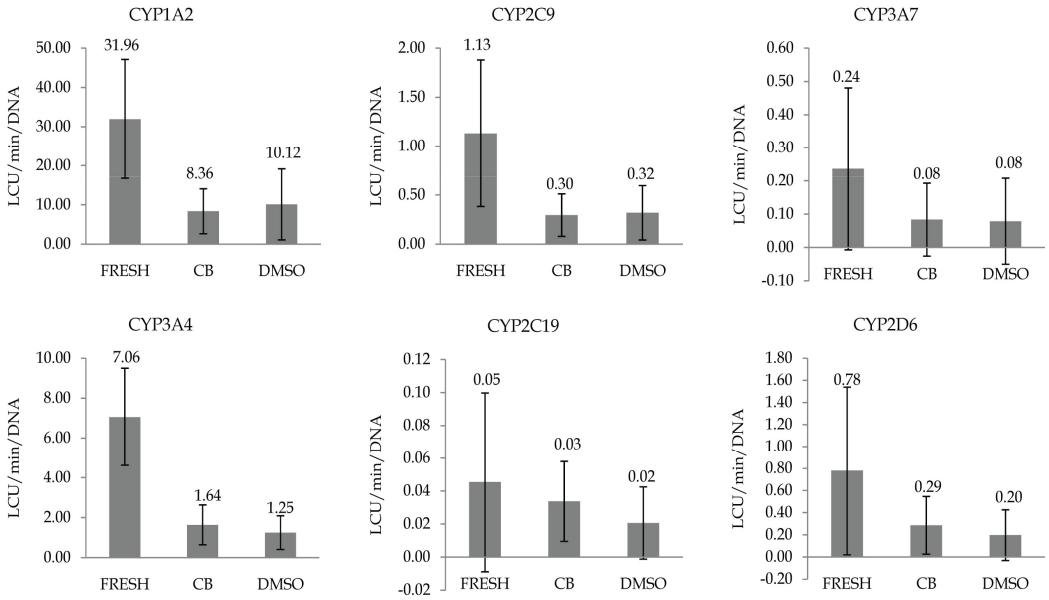

Figure 4.8: Hepatocyte functionality before and after freezing: Activity of the major cytochrome P450 enzymes; CYP1A2, CYP2C9, CYP3A7, CYP3A4, CYP2C19 and CYP2D6 for the fresh hepatocytes (FRESH) compared to hepatocytes cryopreserved using either the new protocol (CB) or the standard DMSO-UW protocol (DMSO). The standard deviation exceeded the mean in many cases illustrating the high within-groups variability. Data is presented as luminescence (LCU) per minute (min) per DNA in nanograms.

4.4 LIVER TISSUE PRESERVES BETTER THAN SIGLE HEPATOCYTES

Data in (paper IV) suggested that cold storage of liver samples as a tissue is superior to preserving them as single hepatocytes.

4.4.1 Viability of Hepatocytes

Cellular viability evaluated by trypan blue exclusion method was higher in UW tissue cells compared to UW cells favoring tissue preservation (figure 4.9).

4.4.2 Apoptosis of Hepatocytes

Results from apoptosis analysis were also in favor of UW tissue cells. Apoptosis rates were similar between fresh and preserved tissues. However, a significant increase in apoptosis rate was noticed in UW cells compared to fresh and preserved tissues and UW tissue cells. This was reflected by significantly higher numbers of TUNEL positive hepatocytes as well as significantly higher caspase activity in UW cells (figure 4.9).

4.4.3 Plating Efficiency

The ability of the hepatocytes to adhere to the culture dish was significantly lower in UW cells compared to the other groups, while it was not significantly different between fresh and UW tissue cells (figure 4.10).

4.4.4 Activity of Drug Metabolizing Enzymes

CYPs activity was not significantly different between UW cells and UW tissue cells, although there was a tendency for activity to be lower in UW cells and to be similar between fresh and UW tissue cells (figure 4.10). CYPs activity was successfully induced in culture, where RIF induced CYP3A4 more than PB (figure 4.11).
Similarly, EROD, resorufin and ammonia assays did not favor UW tissue cells over UW cells (figure 4.11).

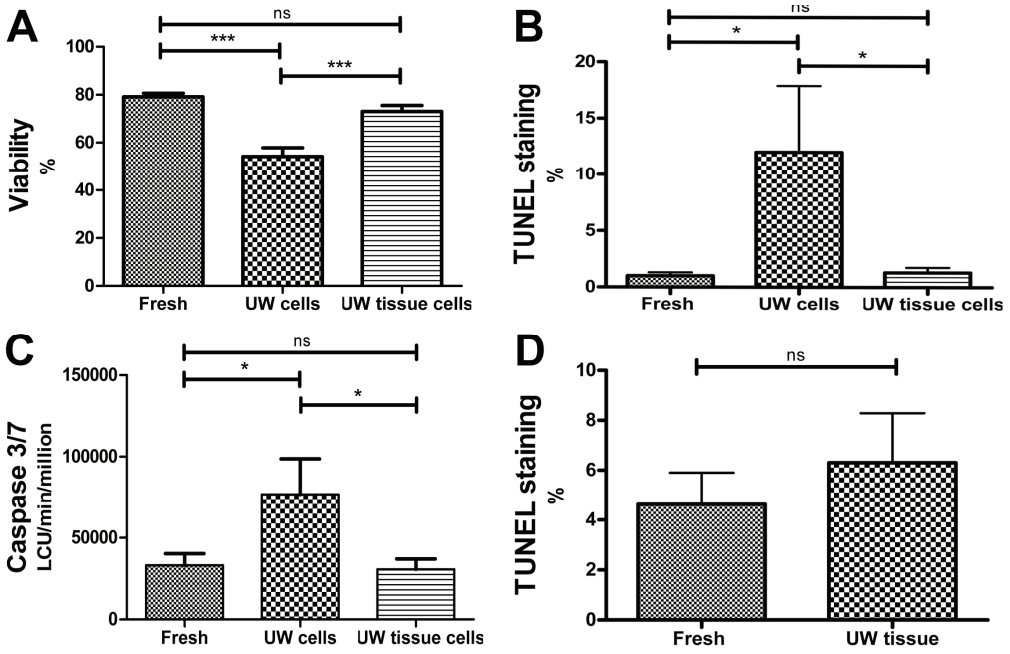

Figure 4.9: **(A)** Hepatocyte viability, **(B)** TUNEL staining and **(C)** Caspase 3/7 activity for hepatocytes directly after isolation (fresh), after storage of single hepatocytes in UW (UW cells), and after repeated hepatocyte isolation (UW tissue cells). **(D)** TUNEL staining was also performed on liver tissue obtained directly after procurement (Fresh) and after storage of liver tissue for 48h in UW (UW tissue). Bars show mean and standard error.

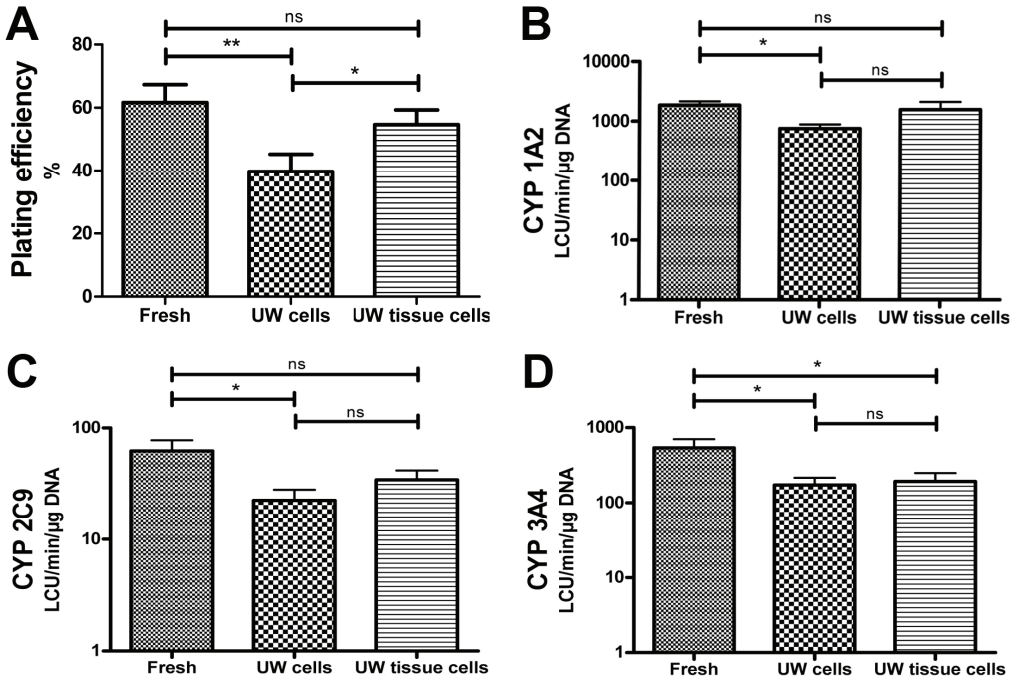

Figure 4.10: **(A)** Plating efficiency and Cytochrome P450 activity for **(B)** CYP1A2, **(C)** CYP2C9, **(D)** CYP3A4 for hepatocytes directly after isolation (fresh), after storage of single hepatocytes in UW (UW cells), and after repeated hepatocyte isolation (UW tissue cells). CYPs activities were measured by luciferin-derived luminescent substrates. Bars show mean and standard error.

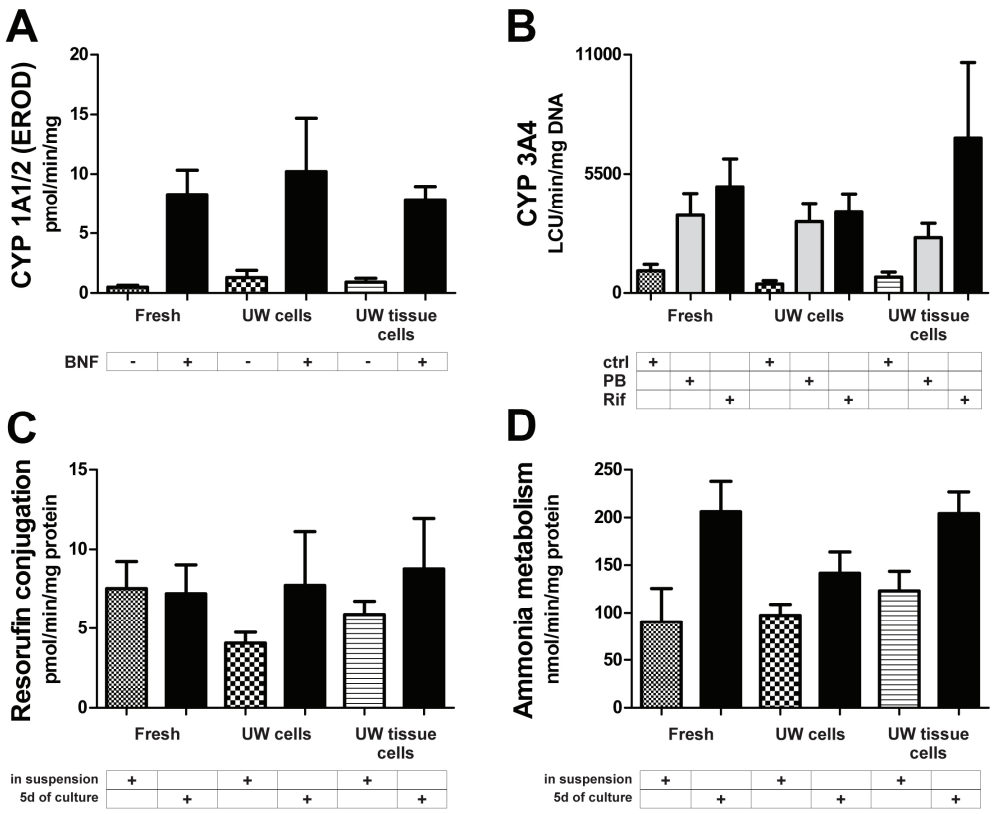

Figure 4.11: **(A)** EROD, **(B)** CYP3A4, **(C)** Resorufin, and **(D)** ammonia assays for hepatocytes directly after isolation (fresh), after storage of single hepatocytes in UW (UW cells), and after repeated hepatocyte isolation (UW tissue cells). Enzyme induction for 72hr was done by β-Naphthoflavone (BNF) (CYP1A1/2) and Rifampicin (Rif) and Phenobarbital (PB) (CYP3A4). Resorufin and ammonia assays were done in suspension and after 5 days in culture. Bars show mean and standard error.

5 GENERAL DISCUSSION

5.1 NEED FOR CELLULAR THERAPY FOR LIVER DISEASE

Liver disease is a major health problem accounting for 4.4% of all deaths worldwide (Mathers, Lopez et al. 2006). In comparison to many other body organs, the liver is imparted a phenomenal capacity to regenerate. It is mainly the fully differentiated liver cells that are responsible for liver regeneration and its mass restoration. However, a role for hepatic and non-hepatic stem cells is strongly supported (Furuyama, Kawaguchi et al. 2011). Unfortunately, liver regeneration is not endless and becomes defective in end-stage liver disease. Hepatocytes lose their regenerative capacity in cirrhosis, obesity and NASH (Farrell, Robertson et al. 2002; Lin, Lim et al. 2010).

The main treatment options available for end-stage liver disease are; orthotopic liver transplantation (OLT), hepatocyte transplantation (HT) and extracorporeal liver support systems. OLT has many limitations, the most serious of which is the unavailability of organ donors with up to 10% of patients dying in the waiting list (Strom and Ellis 2011). HT, despite its promising results, is still an experimental treatment option. Although it makes it possible for other sources of hepatocytes such as porcine and immortalized hepatocytes to be used, human hepatocytes are the most frequent cell type successfully used. Similar to HT, extracorporeal support systems are still under extensive investigation and depend mainly on human hepatocytes in their function.

Stem cells carry a great hope for liver disease patients as they carry the potentiality not only to differentiate to hepatocytes but also to support hepatocyte function when co-transplanted with them (Gomez-Aristizabal, Keating et al. 2009; Synnergren, Heins et al. 2010; Chen, Tseng et al. 2012) (table 5.1). Cellular replacement therapy for liver disease theoretically applies the same basic principles as those for HT when it comes to cell numbers needed, injection sites, mechanism of action, tissue integration and support of liver function. Stem cells are superior to hepatocytes in terms of their high proliferative potential, self-renewal capacity and high differentiation potential, not only to hepatocytes but also to other liver cell types *in vitro* (Cardinale, Wang et al. 2011). This has raised our interest as well as others in improving stem cell differentiation to hepatocytes and studying the potential supportive role for MSC co-transplantation with hepatocytes.

5.2 CHOICE BETWEEN DIFFERENT STEM CELL GROUPS

An ideal stem cell population for liver replacement therapy has not been identified yet. Pluripotent stem cells such as human ESCs and iPSCs have tremendous proliferation and differentiation potentials compared to multipotent stem cells such as MSCs. Pluripotency, on the other hand, needs lots of adjustments in culturing conditions and differentiation protocols to maintain and precisely direct pluripotent stem cells towards a specific cell fate *in vitro* (Suwinska and Ciemerych 2011). iPSCs need more effort, compared to ESCs, in terms of safe and effective pluripotency induction. On the other hand, iPSCs are superior to ESCs when it comes to ethical and immune rejection issues. With iPSCs, pluripotent cells can be created from patient's own cells and then used as patient-specific cellular replacement therapy. It is known that iPSCs keep the same human leukocyte antigen (HLA) genotype of their parental somatic cells (Masaki,

Ishikawa et al. 2007), in addition to having similar proliferation and differentiation potentials as ESCs (Ishikawa, Banas et al. 2012). However, it has been shown recently that iPSCs can induce immune response in syngeneic recipients due to abnormal gene expression in some differentiated iPSCs. This points out the importance of evaluating the immunogenicity of patient's own iPSCs before any clinical use (Zhao, Zhang et al. 2011).

Cell type	Methods	Outcome	Reference
BM-MNCs	Peripheral vein infusion of fresh cells with 6 months follow up	Significant improvement in albumin and protein levels and CP scores	(Terai, Ishikawa et al. 2006)
	Hepatic artery infusion of fresh cells with 1 year follow up	Variable effect on CP score	(Lyra, Soares et al. 2010)
	Peripheral vein infusion of fresh cells with 6 months follow up	Improvement of CP score	(Kim, Park et al. 2010)
	Intrasplenic/intrahepatic injection of differentiated hepatocyte-like cells with 6 months follow up	Significant improvement in albumin and protein levels, ascitis and CP and MELD scores	(Amer, El-Sayed et al. 2011)
BM-MSCs	Peripheral/portal vein infusion of culture-expanded cells with 6 months follow up	Improvement of albumin, creatinine, bilirubin and INR levels and MELD score	(Kharaziha, Hellstrom et al. 2009)
PB-CD34$^+$Cs (G-CSF mobilized)	Hepatic artery/portal vein infusion of fresh cells with 2 months follow up	Improvement of albumin and bilirubin levels	(Gordon, Levicar et al. 2006)
	Hepatic artery/portal vein infusion of fresh cells with 12-18 months follow up	Improvement of bilirubin levels, no tumor formation upon CT scanning and AFP monitoring	(Levicar, Pai et al. 2008)
	Hepatic artery infusion of fresh cells with 3 months follow up	Improvement of CP score, and bilirubin and liver enzymes levels	(Pai, Zacharoulis et al. 2008)
	Hepatic artery/portal vein infusion of expanded and differentiated cells with 12 months follow up	20% mortality, improvement of bilirubin, albumin, INR and liver enzymes levels	(Salama, Zekri et al. 2010)
PB-MNCs (G-CSF mobilized)	Hepatic artery infusion with 6 months follow up	Improvement of CP score, and serum albumin, no effect on bilirubin and liver enzymes levels	(Han, Yan et al. 2008)

Table 5.1: Clinical use of autologous stem/progenitor cells in liver disease. BM: bone marrow, MNCs: mononuclear cells, MSCs: mesenchymal stem cells, PB: peripheral blood, CD34$^+$Cs: CD34 positive cells, G-CSF: granulocyte-colony stimulating factor, CP: Child-Pugh, MELD: model of end stage liver disease, INR: international normalized ratio, CT: computerized tomography, AFP: α-fetoprotein

This is why we are interested in the optimization of stem cell differentiation to hepatocytes and the unraveling of potential roles for miRNAs in hepatocyte development.

MSCs showed their potential, albeit controversial, ability to differentiate to hepatocytes both *in vitro* and *in vivo* (Sato, Araki et al. 2005; Aurich, Sgodda et al. 2009). Our *in vivo* preliminary work showed similar, albeit weak, evidence for successful hFL-MSC homing, integration and differentiation to hepatocyte-like cells in mouse liver. On the other hand, our efforts to differentiate hFL-MSCs *in vitro* (paper I) did not show a convincing evidence for MSC differentiation to functional hepatocytes. Our data are supported by reports concluding that there is no strong evidence for MSC differentiation to hepatocyte-like cells with a broad range of mature hepatocyte

functions (Strom and Ellis 2011; Ishikawa, Banas et al. 2012) debating true MSC trans-differentiation towards hepatic lineages (Gurudutta, Satija et al. 2012).
There is a growing evidence for other important contributions that MSCs might add to the field of liver cellular therapy apart from their possible trans-differentiation to hepatocytes. These contributions are mainly related to MSC paracrine functions rather than a direct role in liver regeneration. Allogeneic MSC transplantation has been suggested to support hepatocyte survival, proliferation and function both *in vivo* and *in vitro* and to decrease the host allogeneic response to transplanted hepatocytes and modulate stellate cell activation in liver cirrhosis causing its regression (Isoda, Kojima et al. 2004; Gomez-Aristizabal, Keating et al. 2009). Promising phase I/II clinical trials strongly support the use of MSCs in patients with decompensated liver cirrhosis (Mohamadnejad, Alimoghaddam et al. 2007; Kharaziha, Hellstrom et al. 2009; Zhang, Lin et al. 2012) and liver failure (Amer, El-Sayed et al. 2011). In paper I, hFL-MSCs isolated by means of CD271 selection proved to be non-immunogenic and immunomodulatory. An immunomodulatory effect by MSCs isolated from human fetal liver can be predicted also from their ability to inhibit lymphocyte proliferation induced by mitogens (Gotherstrom, Ringden et al. 2003). The immunomodulatory characteristics of MSCs may have therapeutic effects. For example, MSCs showed their ability to enhance tissue repair, probably through promoting survival and proliferation of endogenous cells, inducing angiogenesis, inhibiting inflammatory and immune responses and reducing apoptosis (Lee, Oh et al. 2011). The inhibitory effect of hFL-MSCs, shown in paper I, on lymphocyte allogeneic responses in co-cultures of lymphocytes and HepG2 cells, suggests their potential immunosuppressive role in hepatocyte co-transplantation.

5.3 DIFFERENTIATION PROTOCOLS

5.3.1 Cytokine-based Protocols

The core principle in any stem cell differentiation protocol is to emulate the natural *in vivo* scenario of development. Many cell types have been tested for their differentiation potential to hepatocytes; the most prominent of them were hematopoietic stem cells (Almeida-Porada, Zanjani et al. 2010), MSCs (Aurich, Sgodda et al. 2009), ESCs (Synnergren, Heins et al. 2010) and iPSCs (Chen, Tseng et al. 2012). There are several published protocols tailored for each specific stem cell type. These protocols employ treating stem cells mainly with cytokine cocktails, but sometimes with conditioning media (Chen, Dong et al. 2007), or co-culturing them with liver non-parenchymal cells (Soto-Gutierrez, Navarro-Alvarez et al. 2007; Deng, Chen et al. 2008). Although, a combination of all these methods seems to be closer to the natural scenario, it is generally recommended to use a clearly defined differentiation protocol.
Much information is still lacking about hepatocyte development *in vivo*. The possibility for the presence of unidentified compensatory developmental pathways for other well-known pathways cannot be excluded (Gallitzendoerfer, Abouzied et al. 2008). Also, the concept of redundancy of certain growth factors or pathways raises the question of functional redundancy versus functional interaction (Fassler and Meyer 1995; Miller, Ortega et al. 2000).
The current differentiation protocols employ mainly culture conditions, which are quite far from the natural three-dimensional niche for developing endodermal and hepatic cells. Mutual interaction between hepatoblasts and surrounding mesodermal cells

(Margagliotti, Clotman et al. 2008), endothelial cells (Matsumoto, Yoshitomi et al. 2001) and septum transversum (Rossi, Dunn et al. 2001) seems to be crucial for normal hepatic development. Developing liver cells migrates freely and the relationship between them and the surrounding cells is dynamic with continuous change in the spatial relationship between the cells, which in turn creates a continuous change in effecting cytokine combination and their concentrations (Serls, Doherty et al. 2005). Promising three-dimensional differentiation considering the above-mentioned facts have been done but still need further optimization (Ring, Gerlach et al. 2010; Miki, Ring et al. 2011).

Extracellular matrix composition is crucial for proper liver development. Several important components have been identified during liver development (Shiojiri and Sugiyama 2004). Several materials have been used *in vitro* with new factors continuously added to the list aiming at simulating the *in vivo* architecture. Using natural and synthetic biomaterials for maintaining hepatocyte functions is gaining more interest nowadays (Soto-Gutierrez, Kobayashi et al. 2006) and whether this will be successfully applied in stem cell differentiation *in vitro* still needs further evaluation (Soto-Gutierrez, Navarro-Alvarez et al. 2006).

Stem cell death during *in vitro* hepatocyte differentiation may occur and it is usually attributed to selection of cells with higher commitment to the hepatic fate. However, there might be another explanation for this when putting it into the context of the *in vivo* scenario. It has been shown that for their survival, hepatoblasts need to be protected against apoptosis during development (Doi, Marino et al. 1999; Piazzolla, Meissl et al. 2005). Whether this should be applied in the *in vitro* protocols needs further investigation.

The length of a given *in vitro* differentiation protocol is always an issue, and the target is always to keep it short and simple. Most of the hepatocyte differentiation protocols begin with stimulating stem cells to differentiate to definitive endoderm first, passing to hepatocyte induction and ending with hepatocyte maturation; all within few weeks. Comparing this to the *in vivo* liver development, we know that it takes several months with the maturation itself extending even to the postnatal period (Kelley-Loughnane, Sabla et al. 2002). This fact raises the question of how realistic it is to obtain fully mature stem cell-derived hepatocytes *in vitro* within a relatively short period.

The differentiation protocol used here was previously used elsewhere to differentiate adult MSCs to hepatocytes (Campard, Lysy et al. 2008; Lysy, Smets et al. 2008). Although our stimulated hFL-MSCs showed some similarities to what is stated in these protocols, they did not express any hepatocyte-specific CYP activity. Whether it is cell line dependent or that the protocol needs further optimization requires further evaluation. The challenge still facing the field of stem cell differentiation to hepatocytes is not only to generate fully mature hepatic cells, but also to try to arrange them in a three-dimensional architecture forming a bioartificial liver implant.

5.3.2 miRNA-based Differentiation

The miR-290 cluster is abundantly expressed in ESCs representing up to 70% of all miRNAs in undifferentiated mouse ESCs (Marson, Levine et al. 2008). The hsa-miR-372 is orthologous to the mouse miR-294 (Subramanyam, Lamouille et al. 2011), and members of this cluster are mostly involved in the regulation of cell cycle, especially in ESCs (Marson, Levine et al. 2008). Growing evidence strongly suggests the

involvement of miRNAs not only in maintaining ESC pluripotency but also in guiding their differentiation. miRNAs are upregulated after the induction of ESC differentiation to reduce the expression of pluripotency markers and promote differentiation. It has been shown that miR-296 represses Nanog (Tay, Zhang et al. 2008), while miR-145 represses Oct4, Sox2 and Klf4 (Xu, Papagiannakopoulos et al. 2009). Similarly, miR-134 and miR-470 target Nanog, Oct4 and Sox2 (Tay, Zhang et al. 2008) and miR-200c, miR-203 and miR-183 repress Sox2 and Klf4 (Wellner, Schubert et al. 2009).

In addition, miRNAs showed their ability to induce cellular reprogramming. Reprogramming of human fibroblasts was promoted by overexpression of miR-302 cluster and miR-372 together with using Oct3/4, Sox2, c-Myc and Klf4 (Subramanyam, Lamouille et al. 2011). Quite interestingly, overexpression of the miR-302 cluster together with miR-367 successfully reprogrammed mouse and human somatic cells in the absence of other factors in a way that was more rapid and efficient than classical reprogramming using Oct3/4, Sox2, c-Myc and Klf4 (Anokye-Danso, Trivedi et al. 2011). It is not clearly understood how miRNA overexpression promotes or induces reprogramming, but targeting regulators of cell cycle, cell epigenetics or epithelial-mesenchymal transition such as TGFβ pathway could be an explanation (Subramanyam, Lamouille et al. 2011). In addition to the overexpression of ESC-enriched miRNAs, reduced expression of miRNAs enriched in differentiated cells seemed to enhance reprogramming (Yang, Li et al. 2011) possibly through reducing P53 expression (Choi, Lin et al. 2011). Reprogramming using miRNAs appears to be more rapid, efficient and safer than vector-based reprogramming. Whether this approach can be used similarly to create hepatocyte-derived iPSCs needs extensive research.

In addition to their use in pluripotency induction, miRNAs showed ability to induce differentiation. For example, the overexpression of miR-499 induced myocyte differentiation of cardiac stem/progenitor cells both *in vitro* (Sluijter, van Mil et al. 2010) and *in vivo* (Hosoda, Zheng et al. 2011). A potential role for miRNAs during liver development and regeneration has been suggested. More than 40 miRNAs have been identified so far in human liver (Liu, Fan et al. 2010) and their expression patterns were similar to those for ESC-derived hepatocyte-like cells (Kim, Kim et al. 2011). There is evidence for a role of miRNAs during liver development (Hand, Master et al. 2009; Rogler, Levoci et al. 2009), and it has been shown that promoters of several liver-enriched miRNAs have putative binding sites for the liver-enriched transcription factors (LETFs) HNF4α and FoxA2 (Gao, Schug et al. 2011). For example, the expression levels for miR-122, the most abundant miRNA in the liver, positively correlate with those for LETFs in hepatocarcinoma cell lines (Chang, Nicolas et al. 2004). Moreover, the promotor of miR-122 was stimulated by FoxA2, HNF1α, HNF4α and C/EBPα (Xu, He et al. 2010). Quite recently, it has been shown that miR-122 induced the expression of hepatocyte-specific genes and several LETFs, including HNF4α, suggesting the presence of a positive feedback loop formed by miR-122 and HNF4α directing hepatocyte differentiation (Laudadio, Manfroid et al. 2012). Extensive research is still needed to determine whether miR-122, and other liver-specific miRNAs, can be the first building blocks in a miRNA-based differentiation protocol for hepatocytes.

Our work in paper II, together with others, constitutes the first steps towards the identification of liver-specific miRNAs. We managed to profile miRNAs expressed in human fetal and adult livers. Worthy of mentioning is that miR-122 was not

significantly expressed in our microarray data for no apparent reasons. We could link identified miRNAs bioinformatically to many physiological processes related to liver development. However, lots of work is still needed to identify precisely any potential role for individual miRNAs in liver development and the possibility of benefitting from them in stem cell differentiation towards hepatic lineages.

5.4 OBSTACLES TO LIVER CELLULAR THERAPY

5.4.1 Differentiation Efficiency

The main goal for stem cell differentiation towards hepatic lineages is to use the derived hepatocyte-like cells clinically. A given differentiation protocol should be efficient in terms of both quantity and quality. In order to replace 10-20% of human liver mass, around 10^{10} cells would be required (Dan 2012) and the differentiation protocol must result in pure and homogeneous cell population. Characterization should not only focus on expression of hepatic markers on the gene and protein levels, but more on thorough functional evaluation. Functional assessment should focus not only on albumin secretion and CYPs activity, but also on other important hepatic functions such as clotting factor synthesis, detoxification, ammonia metabolism and bilirubin excretion.

In our differentiation experiments, we focused mainly on CYPs activity and not so much on detection of hepatic markers to evaluate the maturity of differentiated stem cells. So far, no convincing evidence supports the presence of real stem cell-derived hepatocytes and further optimization is still needed for the differentiation protocols (Strom and Ellis 2011; Dan 2012; Ishikawa, Banas et al. 2012). Lots of efforts are still needed to improve the MSC (and ESC/iPSC) differentiation protocols. As we have shown in paper I, it may be possible to co-transplant hFL-MSCs directly without differentiation with hepatocytes to support their survival and function and possibly ameliorate host immune response against them.

5.4.2 Safety

Tumor formation is one of the biggest concerns regarding pluripotent stem cell therapy. Also, using multipotent stem cells is not without risk. It has been shown that only one undifferentiated ESC is sufficient to form a teratoma upon transplantation (Ishii, Yasuchika et al. 2007) where uncontrolled replication or epithelial-mesenchymal transition could be possible explanations. Scaling-up and differentiation of stem cells is critical where cells might transform or precipitate genetic mutations. Thus, safety tests need to be carried out before using stem cells for transplantation. Also, stem cells must be cultured under good manufacturing practice (GMP) criteria and xeno-free culturing systems should be applied. Furthermore, the use of immunosuppression carries risk of infection among others. Using autologous iPSCs/MSCs and immune inert MSCs could be useful in this context.

5.4.3 Transplantation, Homing and Repopulation

Cell transplantation can be performed through a catheter introduced into the portal vein, hepatic artery or splenic artery (Puppi and Dhawan 2009). Bleeding, thrombosis, infection and portal hypertension are potential risks (Gagandeep, Rajvanshi et al. 2000). No convincing data support one route to be ideal for transplantation. For hFL-MSC

transplantation, we used the spleen in mouse for technical feasibility. Homing signals, such as stem cell factor, have been reported to help stem cells to home into the liver in animal studies (Hatch, Zheng et al. 2002). Whether this can be enough for stem cell homing to human liver, still needs further assessment. Hepatocyte engraftment is not usually more than 30% (Chandan Guha, S. S. G. et al.), which might explain the fewer number of the human hepatocyte-like cells seen in the mouse liver in our preliminary data. Luckily, a repopulation of 1-5% was enough to correct, at least transiently, liver functional defects functioning as a bridge to OLT (Fox, Chowdhury et al. 1998; Hughes, Mitry et al. 2012). Functional improvement was only temporary probably due to low engraftment, low replicative pressure and cell loss during injection. Repopulation can be improved probably by preconditioning of the liver e.g. by left lobe embolization, partial hepatectomy and radiation or chemical liver injury (Dan 2012). Co-transplantation with other supportive cell types such as MSCs might help improve engraftment and repopulation as well as modulate host immune response (Isoda, Kojima et al. 2004; Gomez-Aristizabal, Keating et al. 2009). Our preliminary data of hFL-MSC transplantation into mice suggested the possibility of hFL-MSCs to differentiate to hepatocyte-like cells within the host liver. Adding to this the immunomodulatory effects that hFL-MSCs showed in paper I, our next step will be to co-transplant hFL-MSCs and hepatocytes in an animal model.

5.4.4 Cryopreservation and Cold Storage

Preservation of primary human hepatocytes and stem cell-derived hepatocyte-like cells necessitates the presence of a fully optimized cryopreservation protocol. To date, there is no fully optimized cryopreservation protocol available. Several protocols have been published with promising results (Rijntjes, Moshage et al. 1986; Dou, de Sousa et al. 1992; Li, Lu et al. 1999; Alexandre, Viollon-Abadie et al. 2002; Hewitt 2010; Terry, Dhawan et al. 2010), but much improvement can still be done such as hepatocyte pre-incubation with anti-oxidants prior to cryopreservation or including non-permeating cryoprotectants in the freezing solution (Stephenne, Najimi et al. 2010). In paper III, we introduced a new experimentally optimized xeno-free cryoprotectant medium (CB) to the field of hepatocyte cryopreservation. Compared to the standard protocol (DMSO-UW), CB contained not only DMSO but also a non-permeating cryoprotectant at carefully tested concentrations (Holm, Strom et al. 2010), which better preserved hepatocyte viability.

During hepatocyte transplantation, only 5% of the liver mass can be infused at one transplantation event. Cell infusion has to be distributed over hours or days to avoid the risk of portal thrombosis (Fox, Chowdhury et al. 1998). Thus, repeated transplantations are necessary to obtain adequate engraftment. As a result, hepatocytes are needed to be cold stored between repeated infusions (Fox, Chowdhury et al. 1998; Stephenne 2006; Fox, Soltys et al. 2011). However, cold storage is not fully optimized with inevitable hepatocyte necrosis and apoptosis (Smets, Chen et al. 2002; Abrahamse, van Runnard Heimel et al. 2003; Gomez-Lechon, Lahoz et al. 2008; Pless, Sauer et al. 2011; Pless-Petig, Singer et al. 2012). Anoikis, a newly described form of apoptosis, affects mainly cells lacking cell anchorage and starts as early as 15 minutes after hepatocyte isolation (Smets, Chen et al. 2002). This raised our interest in comparing cold storage of liver samples as single hepatocytes versus intact tissue. Our data in paper IV strongly

supported tissue preservation against single cell preservation. However, transplantation experiments using animal models will add to our knowledge in this respect.

5.4.5 Functional Evaluation

Evaluating the function of hepatocytes or hepatocyte-like cells before transplantation to liver patients is of paramount importance. Preserving hepatocyte function is a determining factor during the evaluation of a given cryopreservation/cold storage protocol. The high variability between one liver sample and another usually makes it difficult to define the 'normal liver'. There are many reasons for variability, such as genetic polymorphism, gene expression modulation, tissue quality, and tissue handling before and during hepatocyte isolation (Klieber, Torreilles et al. 2010). Hepatocytes vary dramatically in drug-metabolizing enzyme (DME) activity between different samples as noticed from our papers III and IV. This argues against DME sensitivity in favoring one cell sample over the other. This was in line with what others reported where there were no significant differences between fresh and cryopreserved hepatocytes regarding DME activities (Li, Lu et al. 1999). However, cell viability and plating efficiency may be more sensitive. Plating efficiency refers to the ability of the cells to attach themselves to the culture dish and requires intact cell adhesion molecules (Terry, Hughes et al. 2007), and it is suggested to be a marker for engraftment ability and thus is an important quality to maintain (Gramignoli, Green et al. 2011). In (paper IV), we tested the hepatocytes for the plating efficiency, CYPs activity, ammonia metabolism as well as phase-II metabolism.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

- Human fetal liver contains a mesenchymal stem cell population. The hepatic origin, mesenchymal nature and immunomodulatory properties of these cells suggest them as a candidate for cellular replacement therapy for liver disease mainly for their immunomodulatory and supportive properties when co-transplanted with hepatocytes. However, further work is still needed to evaluate the safety, homing, engraftment, survival and function of such cells upon transplantation. When transplanting them into nude mice, we were able to identify human hepatocyte-like cells within mouse liver, but this still needs further evaluation.
- The human liver expresses a wide range of microRNAs, of which many have been linked to liver development. However, functional studies are still needed to verify the specific roles that individual microRNAs account for in liver development and the possibility of their use in microRNA-based stem cell differentiation protocols.
- A new xeno-free cryopreservation medium has been introduced to the field of hepatocyte cryopreservation. The new medium still needs to be tested in experimental animals.
- Cold storage of liver samples as tissue seemed to preserve hepatocyte viability and function better than when preserved as single cells. Further *in vivo* transplantation of preserved tissue hepatocytes is still needed to confirm their better survival and functionality.

7 ACKNOWLEDGEMENTS

And finally this is my thesis! And finally my 'PhD Marathon' is coming to an end! Back in Egypt, my beloved homeland, they call the PhD degree sometimes 'the big degree' *'Elshehada Elkebeera'*. I used not to agree much with this title, as hardworking people will continue to get higher and higher degrees. However, after my relatively long PhD marathon, I admit that it was a big challenge and now see it as a big achievement. During my PhD journey, I worked with many people in many laboratories. I had lots of 'Ups and Downs' and passed lots of tough times where I said to myself 'it is probably the end', but it was not so! Life continued and I managed to collect huge amounts of experience and memories. Now, I see the positive side of it where I sometimes learned from the 'Downs' what I would not learn from the 'Ups'.

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8 REFERENCES

Abdel-Misih, S. R. and M. Bloomston (2010). "Liver anatomy." Surg Clin North Am **90**(4): 643-653.

Abrahamse, S. L., P. van Runnard Heimel, et al. (2003). "Induction of necrosis and DNA fragmentation during hypothermic preservation of hepatocytes in UW, HTK, and Celsior solutions." Cell Transplant **12**(1): 59-68.

Ader, T., R. Norel, et al. (2006). "Transcriptional profiling implicates TGFbeta/BMP and Notch signaling pathways in ductular differentiation of fetal murine hepatoblasts." Mech Dev **123**(2): 177-194.

Alexandre, E., C. Viollon-Abadie, et al. (2002). "Cryopreservation of adult human hepatocytes obtained from resected liver biopsies." Cryobiology **44**(2): 103-113.

Almeida-Porada, G., E. D. Zanjani, et al. (2010). "Bone marrow stem cells and liver regeneration." Exp Hematol **38**(7): 574-580.

Amer, M. E., S. Z. El-Sayed, et al. (2011). "Clinical and laboratory evaluation of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells." Eur J Gastroenterol Hepatol **23**(10): 936-941.

Anokye-Danso, F., C. M. Trivedi, et al. (2011). "Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency." Cell Stem Cell **8**(4): 376-388.

Antoniou, A., P. Raynaud, et al. (2009). "Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9." Gastroenterology **136**(7): 2325-2333.

Anzai, H., A. Kamiya, et al. (2003). "Impaired differentiation of fetal hepatocytes in homozygous jumonji mice." Mech Dev **120**(7): 791-800.

Aurich, H., M. Sgodda, et al. (2009). "Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo." Gut **58**(4): 570-581.

Basma, H., A. Soto-Gutierrez, et al. (2009). "Differentiation and transplantation of human embryonic stem cell-derived hepatocytes." Gastroenterology **136**(3): 990-999.

Bayascas, J. R., V. J. Yuste, et al. (2002). "Isolation of AmphiCASP-3/7, an ancestral caspase from amphioxus (Branchiostoma floridae). Evolutionary considerations for vertebrate caspases." Cell Death Differ **9**(10): 1078-1089.

Benhamouche, S., T. Decaens, et al. (2006). "Apc tumor suppressor gene is the "zonation-keeper" of mouse liver." Dev Cell **10**(6): 759-770.

Bergstrom, R., S. Strom, et al. (2011). "Xeno-free culture of human pluripotent stem cells." Methods Mol Biol **767**: 125-136.

Birraux, J., B. E. Wildhaber, et al. (2010). "Ex vivo hepatocyte gene therapy: increased biosafety protocol for transduction in suspension with lentiviral vectors and immediate transplantation (SLIT)." Transplantation **89**(1): 127-129.

Blanchard, R. L., R. R. Freimuth, et al. (2004). "A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily." Pharmacogenetics **14**(3): 199-211.

Borchert, G. M., W. Lanier, et al. (2006). "RNA polymerase III transcribes human microRNAs." Nat Struct Mol Biol **13**(12): 1097-1101.

Bort, R., M. Signore, et al. (2006). "Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development." Dev Biol **290**(1): 44-56.

Bossard, P. and K. S. Zaret (1998). "GATA transcription factors as potentiators of gut endoderm differentiation." Development **125**(24): 4909-4917.

Brautigam, A., D. Gagneul, et al. (2007). "High-throughput colorimetric method for the parallel assay of glyoxylic acid and ammonium in a single extract." Anal Biochem **362**(1): 151-153.

Brodersen, P. and O. Voinnet (2009). "Revisiting the principles of microRNA target recognition and mode of action." Nat Rev Mol Cell Biol **10**(2): 141-148.

Burke, M. D. and R. T. Mayer (1974). "Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene." Drug Metab Dispos **2**(6): 583-588.
Calmont, A., E. Wandzioch, et al. (2006). "An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells." Dev Cell **11**(3): 339-348.
Campard, D., P. A. Lysy, et al. (2008). "Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells." Gastroenterology **134**(3): 833-848.
Caplan, A. I. and J. E. Dennis (2006). "Mesenchymal stem cells as trophic mediators." J Cell Biochem **98**(5): 1076-1084.
Cardinale, V., Y. Wang, et al. (2011). "Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets." Hepatology **54**(6): 2159-2172.
Chan, J., S. N. Waddington, et al. (2007). "Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse." Stem Cells **25**(4): 875-884.
Chandan Guha, S. S. G., et al. Hepatocyte Transplantation and Liver-Directed Gene Therapy, Landes Bioscience.
Chang, J., E. Nicolas, et al. (2004). "miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1." RNA Biol **1**(2): 106-113.
Chen, J. F., E. M. Mandel, et al. (2006). "The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation." Nat Genet **38**(2): 228-233.
Chen, Y., X. J. Dong, et al. (2007). "In vitro differentiation of mouse bone marrow stromal stem cells into hepatocytes induced by conditioned culture medium of hepatocytes." J Cell Biochem **102**(1): 52-63.
Chen, Y., A. Soto-Gutierrez, et al. (2006). "Instant hepatic differentiation of human embryonic stem cells using activin A and a deleted variant of HGF." Cell Transplant **15**(10): 865-871.
Chen, Y. F., C. Y. Tseng, et al. (2012). "Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol." Hepatology **55**(4): 1193-1203.
Choi, S. S. and A. M. Diehl (2009). "Epithelial-to-mesenchymal transitions in the liver." Hepatology **50**(6): 2007-2013.
Choi, Y. J., C. P. Lin, et al. (2011). "miR-34 miRNAs provide a barrier for somatic cell reprogramming." Nat Cell Biol **13**(11): 1353-1360.
Christ, B. and P. Stock (2012). "Mesenchymal stem cell-derived hepatocytes for functional liver replacement." Frontiers in immunology **3**: 168.
Clotman, F., P. Jacquemin, et al. (2005). "Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors." Genes Dev **19**(16): 1849-1854.
Clotman, F., V. J. Lannoy, et al. (2002). "The onecut transcription factor HNF6 is required for normal development of the biliary tract." Development **129**(8): 1819-1828.
Clotman, F., L. Libbrecht, et al. (2003). "Hepatic artery malformations associated with a primary defect in intrahepatic bile duct development." J Hepatol **39**(5): 686-692.
Coffinier, C., L. Gresh, et al. (2002). "Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta." Development **129**(8): 1829-1838.
Collardeau-Frachon, S. and J. Y. Scoazec (2008). "Vascular development and differentiation during human liver organogenesis." Anat Rec (Hoboken) **291**(6): 614-627.
Compton, S. J. and C. G. Jones (1985). "Mechanism of dye response and interference in the Bradford protein assay." Anal Biochem **151**(2): 369-374.
Corless, J. K. and H. M. Middleton, 3rd (1983). "Normal liver function. A basis for understanding hepatic disease." Arch Intern Med **143**(12): 2291-2294.
Costa, L. G., W. F. Li, et al. (1999). "The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism." Chem Biol Interact **119-120**: 429-438.

Dan, Y. Y. (2012). "Clinical uses of liver stem cells." Methods Mol Biol **826**: 11-23.
de Wildt, S. N., G. L. Kearns, et al. (1999). "Glucuronidation in humans. Pharmacogenetic and developmental aspects." Clin Pharmacokinet **36**(6): 439-452.
Decaens, T., C. Godard, et al. (2008). "Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate." Hepatology **47**(1): 247-258.
Deng, X., Y. X. Chen, et al. (2008). "Hepatic stellate cells modulate the differentiation of bone marrow mesenchymal stem cells into hepatocyte-like cells." J Cell Physiol **217**(1): 138-144.
Doi, T. S., M. W. Marino, et al. (1999). "Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality." Proc Natl Acad Sci U S A **96**(6): 2994-2999.
Dominici, M., K. Le Blanc, et al. (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.
Dou, M., G. de Sousa, et al. (1992). "Thawed human hepatocytes in primary culture." Cryobiology **29**(4): 454-469.
Durrington, P. N., B. Mackness, et al. (2001). "Paraoxonase and atherosclerosis." Arterioscler Thromb Vasc Biol **21**(4): 473-480.
Eberhart, J. K., X. He, et al. (2008). "MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis." Nat Genet **40**(3): 290-298.
Edenberg, H. J. and W. F. Bosron (1997). Comprehensive toxicology. New York, Pergamon.
El-Serag, H. B. (2001). "Epidemiology of hepatocellular carcinoma." Clin Liver Dis **5**(1): 87-107, vi.
Elmen, J., M. Lindow, et al. (2008). "Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver." Nucleic Acids Res **36**(4): 1153-1162.
Enomoto, H., K. Yoshida, et al. (2002). "Hepatoma-derived growth factor is highly expressed in developing liver and promotes fetal hepatocyte proliferation." Hepatology **36**(6): 1519-1527.
Esau, C., S. Davis, et al. (2006). "miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting." Cell Metab **3**(2): 87-98.
Eulalio, A., E. Huntzinger, et al. (2008). "Getting to the root of miRNA-mediated gene silencing." Cell **132**(1): 9-14.
Falagas, M. E., K. Z. Vardakas, et al. (2007). "Under-diagnosis of common chronic diseases: prevalence and impact on human health." Int J Clin Pract **61**(9): 1569-1579.
Farrell, G. C., G. R. Robertson, et al. (2002). "Liver regeneration in obese mice with fatty livers: does the impairment have relevance for other types of fatty liver disease?" Hepatology **35**(3): 731-732.
Fassler, R. and M. Meyer (1995). "Consequences of lack of beta 1 integrin gene expression in mice." Genes Dev **9**(15): 1896-1908.
Fattovich, G., G. Giustina, et al. (1997). "Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients." Gastroenterology **112**(2): 463-472.
Fattovich, G., T. Stroffolini, et al. (2004). "Hepatocellular carcinoma in cirrhosis: incidence and risk factors." Gastroenterology **127**(5 Suppl 1): S35-50.
Filipowicz, W., S. N. Bhattacharyya, et al. (2008). "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" Nat Rev Genet **9**(2): 102-114.
Fitzpatrick, E., R. R. Mitry, et al. (2009). "Human hepatocyte transplantation: state of the art." J Intern Med **266**(4): 339-357.
Fox, I. J., J. R. Chowdhury, et al. (1998). "Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation." N Engl J Med **338**(20): 1422-1426.
Fox, I. J., J. R. Chowdhury, et al. (1998). "Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation." The New England journal of medicine **338**(20): 1422-1426.
Fox, I. J., K. Soltys, et al. (2011). "Hepatocyte Transplantation for Liver Based Metabolic Disorders." Clinical Trials.gov NCT01345578.

Francavilla, A., P. Ove, et al. (1988). "Regulation of liver size and regeneration: importance in liver transplantation." Transplantation proceedings **20**(1 Suppl 1): 494-497.
Friedenstein, A. J., R. K. Chailakhyan, et al. (1987). "Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers." Cell and tissue kinetics **20**(3): 263-272.
Friedenstein, A. J., R. K. Chailakhyan, et al. (1974). "Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo." Transplantation **17**(4): 331-340.
Friedenstein, A. J., N. W. Latzinik, et al. (1982). "Marrow microenvironment transfer by heterotopic transplantation of freshly isolated and cultured cells in porous sponges." Exp Hematol **10**(2): 217-227.
Fuller, B. J., G. J. Morris, et al. (1980). "Functional recovery of isolated rat hepatocytes upon thawing from –196°C." Cryo-Letters **1**(5): 139-146.
Furuyama, K., Y. Kawaguchi, et al. (2011). "Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine." Nature genetics **43**(1): 34-41.
Gagandeep, S., P. Rajvanshi, et al. (2000). "Transplanted hepatocytes engraft, survive, and proliferate in the liver of rats with carbon tetrachloride-induced cirrhosis." J Pathol **191**(1): 78-85.
Gallitzendoerfer, R., M. M. Abouzied, et al. (2008). "Hepatoma-derived growth factor (HDGF) is dispensable for normal mouse development." Dev Dyn **237**(7): 1875-1885.
Gao, Y., J. Schug, et al. (2011). "Tissue-specific regulation of mouse microRNA genes in endoderm-derived tissues." Nucleic Acids Res **39**(2): 454-463.
Gavrieli, Y., Y. Sherman, et al. (1992). "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation." J Cell Biol **119**(3): 493-501.
Gomez-Aristizabal, A., A. Keating, et al. (2009). "Mesenchymal stromal cells as supportive cells for hepatocytes." Mol Ther **17**(9): 1504-1508.
Gomez-Lechon, M. J., A. Lahoz, et al. (2008). "Evaluation of drug-metabolizing and functional competence of human hepatocytes incubated under hypothermia in different media for clinical infusion." Cell Transplant **17**(8): 887-897.
Gonzalez, J. F., R. Reimschuessel, et al. (2009). "Kinetics of hepatic phase I and II biotransformation reactions in eight finfish species." Mar Environ Res **67**(4-5): 183-188.
Gordon, M. Y., N. Levicar, et al. (2006). "Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor." Stem Cells **24**(7): 1822-1830.
Gotherstrom, C., O. Ringden, et al. (2003). "Immunomodulatory effects of human foetal liver-derived mesenchymal stem cells." Bone Marrow Transplant **32**(3): 265-272.
Gotherstrom, C., A. West, et al. (2005). "Difference in gene expression between human fetal liver and adult bone marrow mesenchymal stem cells." Haematologica **90**(8): 1017-1026.
Gouysse, G., A. Couvelard, et al. (2002). "Relationship between vascular development and vascular differentiation during liver organogenesis in humans." J Hepatol **37**(6): 730-740.
Gramignoli, R., M. L. Green, et al. (2011). "Development and application of purified tissue dissociation enzyme mixtures for human hepatocyte isolation." Cell Transplant.
Gramignoli, R., F. Marongiu, et al. (2011). "Isolation and evaluation of human hepatocytes for cell-based therapy." Digestive and Liver Disease **43**: S96-S96.
Graudal, N., P. Leth, et al. (1991). "Characteristics of cirrhosis undiagnosed during life: a comparative analysis of 73 undiagnosed cases and 149 diagnosed cases of cirrhosis, detected in 4929 consecutive autopsies." J Intern Med **230**(2): 165-171.
Greco, D., P. Somervuo, et al. (2008). "Physiology, pathology and relatedness of human tissues from gene expression meta-analysis." PLoS One **3**(4): e1880.

Grisham, J. W. (1962). "A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H3." Cancer research **22**: 842-849.
Guha, I. H. and J. P. Iredale (2007). Clinical and diagnostic aspects of cirrhosis. Oxford, Blackwell Publishing Ltd.
Guillot, P. V., C. Gotherstrom, et al. (2007). "Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC." Stem Cells **25**(3): 646-654.
Gumucio, J. J., C. M. Berkovitz, et al. (1996). Liver and biliary diseases. Baltimore, Williams & Wilkins.
Gurudutta, G. U., N. K. Satija, et al. (2012). "Stem cell therapy: a novel & futuristic treatment modality for disaster injuries." Indian J Med Res **135**: 15-25.
Han, Y., L. Yan, et al. (2008). "Controlled trials in hepatitis B virus-related decompensate liver cirrhosis: peripheral blood monocyte transplant versus granulocyte-colony-stimulating factor mobilization therapy." Cytotherapy **10**(4): 390-396.
Hand, N. J., Z. R. Master, et al. (2009). "The microRNA-30 family is required for vertebrate hepatobiliary development." Gastroenterology **136**(3): 1081-1090.
Hart, S. N., Y. Cui, et al. (2009). "Three patterns of cytochrome P450 gene expression during liver maturation in mice." Drug Metab Dispos **37**(1): 116-121.
Hata, M., M. Nanno, et al. (1993). "Establishment of a hepatocytic epithelial cell line from the murine fetal liver capable of promoting hemopoietic cell proliferation." J Cell Physiol **154**(2): 381-392.
Hatch, H. M., D. Zheng, et al. (2002). "SDF-1alpha/CXCR4: a mechanism for hepatic oval cell activation and bone marrow stem cell recruitment to the injured liver of rats." Cloning Stem Cells **4**(4): 339-351.
Hayes, J. D., J. U. Flanagan, et al. (2005). "Glutathione transferases." Annu Rev Pharmacol Toxicol **45**: 51-88.
He, Z., C. Ehrnfelt, et al. (2004). "Aberrant expression of alpha-Gal on primary human endothelium does not confer susceptibility to NK cell cytotoxicity or increased NK cell adhesion." Eur J Immunol **34**(4): 1185-1195.
Hewitt, N. J. (2010). "Optimisation of the cryopreservation of primary hepatocytes." Methods Mol Biol **640**: 83-105.
Hines, R. N. (2008). "The ontogeny of drug metabolism enzymes and implications for adverse drug events." Pharmacol Ther **118**(2): 250-267.
Holm, F., S. Strom, et al. (2010). "An effective serum- and xeno-free chemically defined freezing procedure for human embryonic and induced pluripotent stem cells." Hum Reprod **25**(5): 1271-1279.
Hosoda, T., H. Zheng, et al. (2011). "Human cardiac stem cell differentiation is regulated by a mircrine mechanism." Circulation **123**(12): 1287-1296.
Hovatta, O., M. Mikkola, et al. (2003). "A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells." Hum Reprod **18**(7): 1404-1409.
Hughes, R. D., R. R. Mitry, et al. (2012). "Current status of hepatocyte transplantation." Transplantation **93**(4): 342-347.
Hunter, M. P., C. M. Wilson, et al. (2007). "The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis." Dev Biol **308**(2): 355-367.
Hussain, S. Z., T. Sneddon, et al. (2004). "Wnt impacts growth and differentiation in ex vivo liver development." Exp Cell Res **292**(1): 157-169.
Ijpenberg, A., J. M. Perez-Pomares, et al. (2007). "Wt1 and retinoic acid signaling are essential for stellate cell development and liver morphogenesis." Dev Biol **312**(1): 157-170.
Inzunza, J., K. Gertow, et al. (2005). "Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells." Stem Cells **23**(4): 544-549.
Ishibashi, H., M. Nakamura, et al. (2009). "Liver architecture, cell function, and disease." Semin Immunopathol **31**(3): 399-409.

Ishii, T., K. Yasuchika, et al. (2007). "Transplantation of embryonic stem cell-derived endodermal cells into mice with induced lethal liver damage." Stem Cells **25**(12): 3252-3260.
Ishikawa, T., A. Banas, et al. (2012). "Regenerative Cells for Transplantation in Hepatic Failure." Cell Transplantation(13): 387-399.
Isoda, K., M. Kojima, et al. (2004). "Maintenance of hepatocyte functions by coculture with bone marrow stromal cells." Journal of bioscience and bioengineering **97**(5): 343-346.
Isoda, K., M. Kojima, et al. (2004). "Maintenance of hepatocyte functions by coculture with bone marrow stromal cells." J Biosci Bioeng **97**(5): 343-346.
Ito, Y., T. Matsui, et al. (2000). "Retroviral gene transfer of signaling molecules into murine fetal hepatocytes defines distinct roles for the STAT3 and ras pathways during hepatic development." Hepatology **32**(6): 1370-1376.
Johnnidis, J. B., M. H. Harris, et al. (2008). "Regulation of progenitor cell proliferation and granulocyte function by microRNA-223." Nature **451**(7182): 1125-1129.
Jones, E. and D. McGonagle (2008). "Human bone marrow mesenchymal stem cells in vivo." Rheumatology (Oxford) **47**(2): 126-131.
Jung, J., M. Zheng, et al. (1999). "Initiation of mammalian liver development from endoderm by fibroblast growth factors." Science **284**(5422): 1998-2003.
Jungermann, K. and N. Katz (1989). "Functional specialization of different hepatocyte populations." Physiol Rev **69**(3): 708-764.
Kamiya, A., T. Kinoshita, et al. (2001). "Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways." FEBS Lett **492**(1-2): 90-94.
Kaniwa, N., K. Kurose, et al. (2005). "Racial variability in haplotype frequencies of UGT1A1 and glucuronidation activity of a novel single nucleotide polymorphism 686C> T (P229L) found in an African-American." Drug Metab Dispos **33**(3): 458-465.
Kaufman, E. J. and E. A. Miska (2010). "The microRNAs of Caenorhabditis elegans." Semin Cell Dev Biol **21**(7): 728-737.
Kelley-Loughnane, N., G. E. Sabla, et al. (2002). "Independent and overlapping transcriptional activation during liver development and regeneration in mice." Hepatology **35**(3): 525-534.
Kennea, N. L., S. N. Waddington, et al. (2009). "Differentiation of human fetal mesenchymal stem cells into cells with an oligodendrocyte phenotype." Cell Cycle **8**(7): 1069-1079.
Kharaziha, P., P. M. Hellstrom, et al. (2009). "Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial." Eur J Gastroenterol Hepatol **21**(10): 1199-1205.
Kim, J. K., Y. N. Park, et al. (2010). "Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis." Cell Transplant **19**(10): 1237-1246.
Kim, N., H. Kim, et al. (2011). "Expression profiles of miRNAs in human embryonic stem cells during hepatocyte differentiation." Hepatol Res **41**(2): 170-183.
Kinoshita, T., T. Sekiguchi, et al. (1999). "Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis." Proc Natl Acad Sci U S A **96**(13): 7265-7270.
Klieber, S., F. Torreilles, et al. (2010). "The use of human hepatocytes to investigate drug metabolism and CYP enzyme induction." Methods Mol Biol **640**: 295-308.
Kobayashi, N., T. Fujiwara, et al. (2000). "Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes." Science **287**(5456): 1258-1262.
Krueger, S. K. and D. E. Williams (2005). "Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism." Pharmacol Ther **106**(3): 357-387.
Kyrmizi, I., P. Hatzis, et al. (2006). "Plasticity and expanding complexity of the hepatic transcription factor network during liver development." Genes Dev **20**(16): 2293-2305.

Lahuna, O., L. Fernandez, et al. (1997). "Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone." Proc Natl Acad Sci U S A **94**(23): 12309-12313.
Laudadio, I., I. Manfroid, et al. (2012). "A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation." Gastroenterology **142**(1): 119-129.
Lavon, N. and N. Benvenisty (2005). "Study of hepatocyte differentiation using embryonic stem cells." J Cell Biochem **96**(6): 1193-1202.
Le Blanc, K., F. Frassoni, et al. (2008). "Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study." Lancet **371**(9624): 1579-1586.
Le Blanc, K., L. Tammik, et al. (2003). "Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex." Scand J Immunol **57**(1): 11-20.
Lee, R. C., R. L. Feinbaum, et al. (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-854.
Lee, R. H., J. Y. Oh, et al. (2011). "Therapeutic factors secreted by mesenchymal stromal cells and tissue repair." J Cell Biochem **112**(11): 3073-3078.
Lee, Y., M. Kim, et al. (2004). "MicroRNA genes are transcribed by RNA polymerase II." EMBO J **23**(20): 4051-4060.
Lee, Y. K. and D. D. Moore (2008). "Liver receptor homolog-1, an emerging metabolic modulator." Front Biosci **13**: 5950-5958.
Lekstrom-Himes, J. and K. G. Xanthopoulos (1998). "Biological role of the CCAAT/enhancer-binding protein family of transcription factors." J Biol Chem **273**(44): 28545-28548.
Lemaigre, F. P. (2009). "Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies." Gastroenterology **137**(1): 62-79.
Leung, A. K. and P. A. Sharp (2007). "microRNAs: a safeguard against turmoil?" Cell **130**(4): 581-585.
Levicar, N., M. Pai, et al. (2008). "Long-term clinical results of autologous infusion of mobilized adult bone marrow derived CD34+ cells in patients with chronic liver disease." Cell Prolif **41 Suppl 1**: 115-125.
Li, A. P., C. Lu, et al. (1999). "Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential." Chem Biol Interact **121**(1): 17-35.
Li, J., G. Ning, et al. (2000). "Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha." Genes Dev **14**(4): 464-474.
Lin, W. R., S. N. Lim, et al. (2010). "The histogenesis of regenerative nodules in human liver cirrhosis." Hepatology **51**(3): 1017-1026.
Lin, Y. S., A. L. Dowling, et al. (2002). "Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism." Mol Pharmacol **62**(1): 162-172.
Liu, D., J. Fan, et al. (2009). "Identification of miRNAs in a liver of a human fetus by a modified method." PLoS One **4**(10): e7594.
Liu, D., J. Fan, et al. (2010). "Quantitative analysis of miRNA expression in several developmental stages of human livers." Hepatol Res **40**(8): 813-822.
Lokmane, L., C. Haumaitre, et al. (2008). "Crucial role of vHNF1 in vertebrate hepatic specification." Development **135**(16): 2777-2786.
Loo, C. K. and X. J. Wu (2008). "Origin of stellate cells from submesothelial cells in a developing human liver." Liver Int **28**(10): 1437-1445.
Loretz, L. J., A. P. Li, et al. (1989). "Optimization of cryopreservation procedures for rat and human hepatocytes." Xenobiotica **19**(5): 489-498.
Ludtke, T. H., V. M. Christoffels, et al. (2009). "Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation." Hepatology **49**(3): 969-978.
Lyra, A. C., M. B. Soares, et al. (2010). "Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of

liver function in patients with chronic liver disease: a pilot randomized controlled study." Eur J Gastroenterol Hepatol **22**(1): 33-42.
Lysy, P. A., F. Smets, et al. (2008). "Leukemia inhibitory factor contributes to hepatocyte-like differentiation of human bone marrow mesenchymal stem cells." Differentiation **76**(10): 1057-1067.
Margagliotti, S., F. Clotman, et al. (2008). "Role of metalloproteinases at the onset of liver development." Dev Growth Differ **50**(5): 331-338.
Marson, A., S. S. Levine, et al. (2008). "Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells." Cell **134**(3): 521-533.
Martin, M. A. and M. Bhatia (2005). "Analysis of the human fetal liver hematopoietic microenvironment." Stem Cells Dev **14**(5): 493-504.
Martinez-Hernandez, A. and P. S. Amenta (1995). "The extracellular matrix in hepatic regeneration." FASEB J **9**(14): 1401-1410.
Martinez Barbera, J. P., M. Clements, et al. (2000). "The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation." Development **127**(11): 2433-2445.
Masaki, H., T. Ishikawa, et al. (2007). "Heterogeneity of pluripotent marker gene expression in colonies generated in human iPS cell induction culture." Stem Cell Res **1**(2): 105-115.
Mathers, C., A. Lopez, et al. (2006). The burden of disease and mortality by condition: data, methods, and results for 2001. Washington (DC), Oxford University Press and the World Bank.
Matsumoto, K., R. Miki, et al. (2008). "Wnt9a secreted from the walls of hepatic sinusoids is essential for morphogenesis, proliferation, and glycogen accumulation of chick hepatic epithelium." Dev Biol **319**(2): 234-247.
Matsumoto, K., H. Yoshitomi, et al. (2001). "Liver organogenesis promoted by endothelial cells prior to vascular function." Science **294**(5542): 559-563.
McDaniell, R., D. M. Warthen, et al. (2006). "NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway." Am J Hum Genet **79**(1): 169-173.
Michalopoulos, G. K. and M. C. DeFrances (1997). "Liver regeneration." Science **276**(5309): 60-66.
Miki, T., A. Ring, et al. (2011). "Hepatic differentiation of human embryonic stem cells is promoted by three-dimensional dynamic perfusion culture conditions." Tissue Eng Part C Methods **17**(5): 557-568.
Miller, D. L., S. Ortega, et al. (2000). "Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice." Mol Cell Biol **20**(6): 2260-2268.
Mohamadnejad, M., K. Alimoghaddam, et al. (2007). "Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis." Arch Iran Med **10**(4): 459-466.
Morisseau, C. and B. D. Hammock (2005). "Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles." Annu Rev Pharmacol Toxicol **45**: 311-333.
Mouta Carreira, C., S. M. Nasser, et al. (2001). "LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis." Cancer Res **61**(22): 8079-8084.
Murray, C. J. and A. D. Lopez (1997). "Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study." Lancet **349**(9064): 1498-1504.
Nagata, H., M. Ito, et al. (2003). "Treatment of cirrhosis and liver failure in rats by hepatocyte xenotransplantation." Gastroenterology **124**(2): 422-431.
Nebert, D. W. and V. Vasiliou (2004). "Analysis of the glutathione S-transferase (GST) gene family." Hum Genomics **1**(6): 460-464.
Nishina, H., C. Vaz, et al. (1999). "Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4." Development **126**(3): 505-516.

Nonaka, H., M. Tanaka, et al. (2007). "Development of murine hepatic sinusoidal endothelial cells characterized by the expression of hyaluronan receptors." Dev Dyn **236**(8): 2258-2267.
Odom, D. T., N. Zizlsperger, et al. (2004). "Control of pancreas and liver gene expression by HNF transcription factors." Science **303**(5662): 1378-1381.
Ogawa, K., A. Medline, et al. (1979). "Sequential analysis of hepatic carcinogenesis: the comparative architecture of preneoplastic, malignant, prenatal, postnatal and regenerating liver." Br J Cancer **40**(5): 782-790.
Okita, K., Y. Matsumura, et al. (2011). "A more efficient method to generate integration-free human iPS cells." Nat Methods **8**(5): 409-412.
Overturf, K., M. Al-Dhalimy, et al. (1999). "The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion." The American journal of pathology **155**(6): 2135-2143.
Pai, M., D. Zacharoulis, et al. (2008). "Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis." Am J Gastroenterol **103**(8): 1952-1958.
Paradis, V., N. Youssef, et al. (2001). "Replicative senescence in normal liver, chronic hepatitis C, and hepatocellular carcinomas." Hum Pathol **32**(3): 327-332.
Parkin, D. M., F. Bray, et al. (2001). "Estimating the world cancer burden: Globocan 2000." Int J Cancer **94**(2): 153-156.
Pasquinelli, A. E., B. J. Reinhart, et al. (2000). "Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA." Nature **408**(6808): 86-89.
Perera, M. T., D. F. Mirza, et al. (2009). "Liver transplantation: Issues for the next 20 years." J Gastroenterol Hepatol **24 Suppl 3**: S124-131.
Perez-Pomares, J. M., R. Carmona, et al. (2004). "Contribution of mesothelium-derived cells to liver sinusoids in avian embryos." Dev Dyn **229**(3): 465-474.
Perincheri, S., R. W. Dingle, et al. (2005). "Hereditary persistence of alpha-fetoprotein and H19 expression in liver of BALB/cJ mice is due to a retrovirus insertion in the Zhx2 gene." Proc Natl Acad Sci U S A **102**(2): 396-401.
Petersen, B. E., W. C. Bowen, et al. (1999). "Bone marrow as a potential source of hepatic oval cells." Science **284**(5417): 1168-1170.
Petkov, P. M., J. Zavadil, et al. (2004). "Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays." Hepatology **39**(3): 617-627.
Piazzolla, D., K. Meissl, et al. (2005). "Raf-1 sets the threshold of Fas sensitivity by modulating Rok-alpha signaling." J Cell Biol **171**(6): 1013-1022.
Pierreux, C. E., J. Stafford, et al. (1999). "Antiglucocorticoid activity of hepatocyte nuclear factor-6." Proc Natl Acad Sci U S A **96**(16): 8961-8966.
Pless-Petig, G., B. B. Singer, et al. (2012). "Cold storage of rat hepatocyte suspensions for one week in a customized cold storage solution--preservation of cell attachment and metabolism." PLoS One **7**(7): e40444.
Pless, G., I. M. Sauer, et al. (2011). "Improvement of the cold storage of isolated human hepatocytes." Cell Transplant.
Pless, G., I. M. Sauer, et al. (2012). "Improvement of the cold storage of isolated human hepatocytes." Cell Transplant **21**(1): 23-37.
Potten, C. S. and M. Loeffler (1990). "Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt." Development **110**(4): 1001-1020.
Puppi, J. and A. Dhawan (2009). "Human hepatocyte transplantation overview." Methods Mol Biol **481**: 1-16.
Rabes, H. M., R. Wirsching, et al. (1976). "Analysis of cell cycle compartments of hepatocytes after partial hepatecomy." Cell and tissue kinetics **9**(6): 517-532.
Rappaport, A. M., Z. J. Borowy, et al. (1954). "Subdivision of hexagonal liver lobules into a structural and functional unit; role in hepatic physiology and pathology." Anat Rec **119**(1): 11-33.
Reinhart, B. J., F. J. Slack, et al. (2000). "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans." Nature **403**(6772): 901-906.
Rijntjes, P. J., H. J. Moshage, et al. (1986). "Cryopreservation of adult human hepatocytes. The influence of deep freezing storage on the viability, cell

seeding, survival, fine structures and albumin synthesis in primary cultures." J Hepatol **3**(1): 7-18.
Ring, A., J. Gerlach, et al. (2010). "Hepatic maturation of human fetal hepatocytes in four-compartment three-dimensional perfusion culture." Tissue Eng Part C Methods **16**(5): 835-845.
Rockey, D. C. and S. L. Friedman (2006). Hepatic fibrosis and cirrhosis. New York, Elsevier.
Rodin, S., A. Domogatskaya, et al. (2010). "Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511." Nat Biotechnol **28**(6): 611-615.
Rogler, C. E., L. Levoci, et al. (2009). "MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads." Hepatology **50**(2): 575-584.
Romero-Gallo, J., E. G. Sozmen, et al. (2005). "Inactivation of TGF-beta signaling in hepatocytes results in an increased proliferative response after partial hepatectomy." Oncogene **24**(18): 3028-3041.
Rosa, A., F. M. Spagnoli, et al. (2009). "The miR-430/427/302 family controls mesendodermal fate specification via species-specific target selection." Dev Cell **16**(4): 517-527.
Rossi, J. M., N. R. Dunn, et al. (2001). "Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm." Genes Dev **15**(15): 1998-2009.
Sakaguchi, T. F., K. C. Sadler, et al. (2008). "Endothelial signals modulate hepatocyte apicobasal polarization in zebrafish." Curr Biol **18**(20): 1565-1571.
Salama, H., A. R. Zekri, et al. (2010). "Autologous hematopoietic stem cell transplantation in 48 patients with end-stage chronic liver diseases." Cell Transplant **19**(11): 1475-1486.
Sato, Y., H. Araki, et al. (2005). "Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion." Blood **106**(2): 756-763.
Schwartz, R. E., M. Reyes, et al. (2002). "Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells." J Clin Invest **109**(10): 1291-1302.
Sekine, S., R. Ogawa, et al. (2009). "Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis." Gastroenterology **136**(7): 2304-2315 e2301-2304.
Serls, A. E., S. Doherty, et al. (2005). "Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung." Development **132**(1): 35-47.
Sgroi, A., V. Serre-Beinier, et al. (2009). "What clinical alternatives to whole liver transplantation? Current status of artificial devices and hepatocyte transplantation." Transplantation **87**(4): 457-466.
Shimada, T., H. Yamazaki, et al. (1994). "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 **270**(1): 414-423.
Shin, D., C. H. Shin, et al. (2007). "Bmp and Fgf signaling are essential for liver specification in zebrafish." Development **134**(11): 2041-2050.
Shiojiri, N. and Y. Sugiyama (2004). "Immunolocalization of extracellular matrix components and integrins during mouse liver development." Hepatology **40**(2): 346-355.
Si-Tayeb, K., F. P. Lemaigre, et al. (2010). "Organogenesis and development of the liver." Dev Cell **18**(2): 175-189.
Si-Tayeb, K., F. K. Noto, et al. (2010). "Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells." Hepatology **51**(1): 297-305.
Sim, S. C., R. J. Edwards, et al. (2005). "CYP3A7 protein expression is high in a fraction of adult human livers and partially associated with the CYP3A7*1C allele." Pharmacogenet Genomics **15**(9): 625-631.
Siomi, H. and M. C. Siomi (2010). "Posttranscriptional regulation of microRNA biogenesis in animals." Mol Cell **38**(3): 323-332.

Sivertsson, L., J. Synnergren, et al. (2012). "Hepatic Differentiation and Maturation of Human Embryonic Stem Cells Cultured in a Perfused Three-Dimensional Bioreactor." Stem Cells Dev.
Sluijter, J. P., A. van Mil, et al. (2010). "MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells." Arterioscler Thromb Vasc Biol **30**(4): 859-868.
Smets, F. N., Y. Chen, et al. (2002). "Loss of cell anchorage triggers apoptosis (anoikis) in primary mouse hepatocytes." Mol Genet Metab **75**(4): 344-352.
Song, G., A. D. Sharma, et al. (2010). "MicroRNAs control hepatocyte proliferation during liver regeneration." Hepatology **51**(5): 1735-1743.
Song, Z., J. Cai, et al. (2009). "Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells." Cell Res **19**(11): 1233-1242.
Soto-Gutierrez, A., N. Kobayashi, et al. (2006). "Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes." Nat Biotechnol **24**(11): 1412-1419.
Soto-Gutierrez, A., N. Navarro-Alvarez, et al. (2006). "Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric." Cell Transplant **15**(4): 335-341.
Soto-Gutierrez, A., N. Navarro-Alvarez, et al. (2007). "Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines." Nat Protoc **2**(2): 347-356.
Stanulovic, V. S., I. Kyrmizi, et al. (2007). "Hepatic HNF4alpha deficiency induces periportal expression of glutamine synthetase and other pericentral enzymes." Hepatology **45**(2): 433-444.
Stephenne, X. (2006). "Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency." Gastroenterology **130**: 1317-1323.
Stephenne, X., M. Najimi, et al. (2010). "Hepatocyte cryopreservation: is it time to change the strategy?" World J Gastroenterol **16**(1): 1-14.
Stevens, J. C., R. N. Hines, et al. (2003). "Developmental expression of the major human hepatic CYP3A enzymes." J Pharmacol Exp Ther **307**(2): 573-582.
Stocker, E. and W. D. Heine (1971). "Regeneration of liver parenchyma under normal and pathological conditions." Beitrage zur Pathologie **144**(4): 400-408.
Strom, S., J. Inzunza, et al. (2007). "Mechanical isolation of the inner cell mass is effective in derivation of new human embryonic stem cell lines." Hum Reprod **22**(12): 3051-3058.
Strom, S. C. and E. C. S. Ellis (2011). Cell Therapy of Liver Disease: From Hepatocytes to Stem Cells. London, Burlington, San Diego, Academic Press.
Subramanyam, D., S. Lamouille, et al. (2011). "Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells." Nat Biotechnol **29**(5): 443-448.
Suksaweang, S., C. M. Lin, et al. (2004). "Morphogenesis of chicken liver: identification of localized growth zones and the role of beta-catenin/Wnt in size regulation." Dev Biol **266**(1): 109-122.
Suwinska, A. and M. A. Ciemerych (2011). "Factors regulating pluripotency and differentiation in early mammalian embryos and embryo-derived stem cells." Vitam Horm **87**: 1-37.
Suzuki, A., A. Iwama, et al. (2003). "Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells." Development **130**(11): 2513-2524.
Suzuki, T., Y. Kanai, et al. (2006). "Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development." Mol Cell Biol **26**(16): 6149-6156.
Synnergren, J., N. Heins, et al. (2010). "Transcriptional profiling of human embryonic stem cells differentiating to definitive and primitive endoderm and further toward the hepatic lineage." Stem Cells Dev **19**(7): 961-978.
Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-676.

Tanaka, E., M. Terada, et al. (2000). "Cytochrome P450 2E1: its clinical and toxicological role." J Clin Pharm Ther **25**(3): 165-175.
Tanimizu, N. and A. Miyajima (2004). "Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors." J Cell Sci **117**(Pt 15): 3165-3174.
Tay, Y., J. Zhang, et al. (2008). "MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation." Nature **455**(7216): 1124-1128.
Terai, S., T. Ishikawa, et al. (2006). "Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy." Stem Cells **24**(10): 2292-2298.
Teratani, T., H. Yamamoto, et al. (2005). "Direct hepatic fate specification from mouse embryonic stem cells." Hepatology **41**(4): 836-846.
Terry, C., A. Dhawan, et al. (2010). "Optimization of the cryopreservation and thawing protocol for human hepatocytes for use in cell transplantation." Liver Transpl **16**(2): 229-237.
Terry, C., R. D. Hughes, et al. (2007). "Cryopreservation-induced nonattachment of human hepatocytes: role of adhesion molecules." Cell Transplant **16**(6): 639-647.
Thompson, H. J., R. Strange, et al. (1992). "Apoptosis in the genesis and prevention of cancer." Cancer Epidemiol Biomarkers Prev **1**(7): 597-602.
Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145-1147.
Tremblay, K. D. and K. S. Zaret (2005). "Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues." Dev Biol **280**(1): 87-99.
Tukey, R. H. and C. P. Strassburg (2000). "Human UDP-glucuronosyltransferases: metabolism, expression, and disease." Annu Rev Pharmacol Toxicol **40**: 581-616.
Tzur, G., A. Israel, et al. (2009). "Comprehensive gene and microRNA expression profiling reveals a role for microRNAs in human liver development." PLoS One **4**(10): e7511.
Tzur, G., A. Levy, et al. (2008). "MicroRNA expression patterns and function in endodermal differentiation of human embryonic stem cells." PLoS One **3**(11): e3726.
Van Eyken, P., R. Sciot, et al. (1988). "The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study." Hepatology **8**(6): 1586-1595.
Vasudevan, S., Y. Tong, et al. (2007). "Switching from repression to activation: microRNAs can up-regulate translation." Science **318**(5858): 1931-1934.
Wan, Z., X. G. Zhang, et al. (2012). "Therapeutic liver repopulation for metabolic liver diseases: Advances from bench to bedside." Hepatol Res.
Weinstein, M., S. P. Monga, et al. (2001). "Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development." Mol Cell Biol **21**(15): 5122-5131.
Wellner, U., J. Schubert, et al. (2009). "The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs." Nat Cell Biol **11**(12): 1487-1495.
Wiemann, S. U., A. Satyanarayana, et al. (2002). "Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis." FASEB J **16**(9): 935-942.
Wightman, B., I. Ha, et al. (1993). "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans." Cell **75**(5): 855-862.
Williams, J. A., R. Hyland, et al. (2004). "Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios." Drug Metab Dispos **32**(11): 1201-1208.
Xiao, C., D. P. Calado, et al. (2007). "MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb." Cell **131**(1): 146-159.

Xie, Z., H. Zhang, et al. (2008). "Zinc finger protein ZBTB20 is a key repressor of alpha-fetoprotein gene transcription in liver." Proc Natl Acad Sci U S A **105**(31): 10859-10864.
Xu, H., J. H. He, et al. (2010). "Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development." Hepatology **52**(4): 1431-1442.
Xu, N., T. Papagiannakopoulos, et al. (2009). "MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells." Cell **137**(4): 647-658.
Yamasaki, H., A. Sada, et al. (2006). "Suppression of C/EBPalpha expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression." Development **133**(21): 4233-4243.
Yanai, M., N. Tatsumi, et al. (2008). "FGF signaling segregates biliary cell-lineage from chick hepatoblasts cooperatively with BMP4 and ECM components in vitro." Dev Dyn **237**(5): 1268-1283.
Yang, C. S., Z. Li, et al. (2011). "microRNAs modulate iPS cell generation." RNA **17**(8): 1451-1460.
Yarmush, M. L., J. C. Dunn, et al. (1992). "Assessment of artificial liver support technology." Cell Transplant **1**(5): 323-341.
Yi, R., M. N. Poy, et al. (2008). "A skin microRNA promotes differentiation by repressing 'stemness'." Nature **452**(7184): 225-229.
Yoshimura, A., M. Ichihara, et al. (1996). "Mouse oncostatin M: an immediate early gene induced by multiple cytokines through the JAK-STAT5 pathway." EMBO J **15**(5): 1055-1063.
Yu, J., M. A. Vodyanik, et al. (2007). "Induced pluripotent stem cell lines derived from human somatic cells." Science **318**(5858): 1917-1920.
Zaret, K. S. (2008). "Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation." Nat Rev Genet **9**(5): 329-340.
Zhang, Z., H. Lin, et al. (2012). "Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients." J Gastroenterol Hepatol **27 Suppl 2**: 112-120.
Zhao, T., Z. N. Zhang, et al. (2011). "Immunogenicity of induced pluripotent stem cells." Nature **474**(7350): 212-215.
Zong, Y., A. Panikkar, et al. (2009). "Notch signaling controls liver development by regulating biliary differentiation." Development **136**(10): 1727-1739.