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Experimental models with specific approaches to augment human fetal liver cell engraftment

Akademisk avhandling

som för avläggande av medicine doktorsexamen vid Sahlgrenska akademin, Göteborgs universitet kommer att offentligen försvaras i Conferencecentrum Wallenberg, Lyktan Hall, Medicinaregatan 20A.
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av

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M.V.Sc. (Veterinary Surgery & Radiology)

Fakultetsopponent:
Prof. John Fung
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This thesis is based on the following papers:

I. Fetal liver-derived mesenchymal stromal cells augment engraftment of transplanted hepatocytes.

Meghnad Joshi, Pradeep B. Patil, Zhong He, Jan Holgersson, Michael Olausson, Suchitra Sumitran-Holgersson. *Cytotherapy*, 2012; 14(6): 657-669. (*Published*)

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Abstract

Experimental models with specific approaches to augment human fetal liver cell engraftment

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Background: Liver disease is a common cause of morbidity and mortality worldwide. Orthotopic liver transplantation has so far been the only available therapy for patients with end-stage liver failure. Unfortunately, the availability of donor organs is limited and more than 40% of patients become too sick to survive each year while waiting for liver transplants. Cellular therapy with stem cells and their progeny is a promising new approach to this largely unmet medical need, but is yet to be integrated into the current clinical system. Impediments in cell transplantation are well characterized, but there is lack of reliable solutions, which has limited the use of this technique to act as a bridge (temporary support) to transplantation.

Aims: Studies covered under the current thesis are focused on validation and evaluation of reliable cell sources and feasible protocols for enhancing their engraftment and proliferation in animal models.

Materials and methods: The mammalian fetal liver contains colony-forming cells with high proliferative potential. The use of human fetal liver cells (hFLCs) is a suitable candidate for the purpose of cell therapy and diagnostics. We have evaluated hFLCs lines as a potential source of stem cells and tested their *in vivo* functions in a model of liver injury using nude mouse.

Results and discussion: This thesis has shown that the regimens of preconditioning (using chemokines) or the co-transplantation (liver cells with mesenchymal stem cells) have the possibility to augment engraftment. Also, manipulating liver cells *ex vivo* to increase longevity helps in growing cell colonies much faster for many passages to produce a limitless population. It also demonstrates a novel marker to isolate adult or fetal liver stellate cells, which has an important role in immunoregulation and liver fibrosis.

Summary: This thesis describes and highlights novel and feasible approaches in liver cell transplantation, with the possibility to improve current clinical protocols.

Keywords: cell transplantation, chemokines, SV40, stellate cell, MSCs

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Erratum

Thesis

1. Page 2 – line 9th
   …which stole… Should be **….who stole….**
2. Page 5 – para 3rd :Materials & methods: line 3rd
   ….evaluated hFLCs.. Should be **…evaluated hFLC….**
3. Page 6 – Paper IV: line 4th
   (Manuscript Submitted) Should be **(*In press* – Manuscript ID: CYTH-2013-0257.R1)**
4. Page 10 – line 18th
   Can lentivirus… Should be **Can polyomavirus….**
5. Page13 – before line 1st
   Addition of **SC – Stem cells**
6. Page 22 –line 1st
   …pluripotent cells… Should be **…pluripotent stem cells…**
7. Page 24 – para 2nd : line 7
   …one low is… Should be **…one lobe is…**
8. Page 25 – para 2nd : line 4th
   …state have …. Should be **….status have….**
9. Page 25 – para 2nd : line 9th
   Despite their…. Should be **Despite the….**
10. Page 29 – para 2nd : line 11th
    …survival form… Should be **….survival from…**
11. Page 40 – line 2nd
    …sections of…. Should be **….section of …**
12. Page 41 – para 3rd : line 2nd
    …hepatocytes were… Should be **…hepatocytes (Paper I-IV) were…**
13. Page 44 – para 1st : line 7th
    ..(5, 10, 10 ng/ml).. Should be **…(5, 10, 20 ng/ml)…**
14. Page 44 – para 2nd : line 2nd
    …using four different… Should be **…using different…**
15. Page 44 – para 3rd : line 5th
    …antigen in quenstion. Should be **…antigen of interest.**
16. Page 46 – para 2nd : line 10th
    …(Paper I)… Should be **….(Paper II)….**
17. Page 49 – para 2nd : line 4th
    …recipient since… Should be **…recipient and…**
18. Page 50 – line 4th
    …USA. Should be **…USA respectively.**
19. Page 50 – line 5th
    ..cycle maintained by .. Should be **…cycle by…**
20. Page 68 – line 7th
    Can lentivirus… Should be **Can polyomavirus….**
21. Page 69 – para 2nd : line 9th
    ..useful for the… Should be **…useful in the…**

Erratum

Papers

Paper III

1. Page 7 – line 19th
   …CXCL11 (5, 10, and 10 ng/ml)… Should be **…CXCL11 (5, 10, and 20 ng/ml)…**
2. Paper 14 - line 17th
   ….HNF-4□ , cEBP-□ , …. Should be **…HNF-4α , cEBP-α ,..**
3. Page 15 – line 1st
   …., HNF-4□ , cEBP-□…. Should be **….HNF-4α , cEBP-α ,…..**
4. Page 15 – line 5th
   …. HNF-4□ , cEBP-β ……. Should be **….HNF-4α , cEBP-β ……**
5. Page 18 – line 7th
   ….fetoprotein, CK19, hepatocyte specific … Should be **….fetoprotein, hepatocyte specific…**
6. Page 19 – line 16th-19th
   … Interestingly, engraftment was not proportional to the concentration of chemokines injected, since animals receiving highest levels of the chemokines did not have increased number of engrafted cells. This indicates that the right levels of chemokines are required for efficient engrafment. **This paragraph should be deleted**.
7. Figure S1
   hFL4TERT… Should be **hFL161/hTERT….**

Paper IV

1. Page 11 – line 7th
   Isolation of CD217+…… Should be **Isolation of CD271+….**
2. Page 16 – line 2nd
   …..longer than CD217+ cells. Should be **…….longer than 271+ cells.**

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Pradeep Bhatu Patil

2014

UNIVERSITY OF GOTHENBURG

Laboratory for Transplantation Biology and Regenerative Medicine

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A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or manuscripts are at various stages (in press, submitted, or in manuscript)

Front cover illustration: Pictorial illustration of liver regenerative capacity in ancient story of Zeus and Prometheus.

(http://www.squidoo.com/ghoststoriesfromaroundtheworld)

Painting by Jacob Jordaens, ca. 1640 (Public domain)

In Greek mythology, Prometheus was a Titan known for his wily intelligence, which stole fire from Zeus and gave it to mortals for their use. Zeus then eternally punished him for his offense by being chained to a rock where a predator (or an eagle) would peck out his liver, only to be regenerated, due to his immortality, by dark. Years later the Greek hero Heracles would shoot the vulture (or eagle) and free Prometheus from his chains. Curiously, the liver is the only human internal organ that actually can regenerate itself to a significant extent; Greeks might have prior knowledge of this, which resulted into survivals in battle.

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Whatever is yours today, was somebody else's yesterday and will belong to somebody else's tomorrow.

Change is the law of this universe..... Bhagvat Geeta Saar

ABSTRACT

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List of publications

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Short title: Co-transplantation of hFHs with mesenchymal cell

Paper II: Phenotypic and *in vivo* functional characterization of immortalized human fetal liver cells.

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Short title: SV40-LT transduced hFLCs

Paper III: Chemokine mediated robust augmentation of liver engraftment - A novel approach.

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Short title: hTERT transduced hFLCs and chemokines

Paper IV: CD271 identifies functional human hepatic stellate cells, which localize in perisinusoidal and portal areas in livers after partial hepatectomy

Pradeep B. Patil*, Meghnad Joshi*, Liza Johannesson, Michael Olausson and Suchitra Sumitran-Holgersson. *(Manuscript submitted)*

Short title: human liver stellate cell

* Authors contributed equally to this paper

Populärvetenskaplig sammanfattning

Bakgrund: Leversjukdom är en relativt vanlig och allvarlig åkomma. Obehandlade leversviktspatienter riskerar att avlida om de inte kan erbjudas en levertransplantation. Tyvärr är tillgången på donatororgan begränsad och mer än 40% av patienterna som dör varje år väntar på att få ett lämpligt organ donerat. Stamcellsterapi, eller användandet av andra mer specialiserade celler, skulle eventuellt kunna användas som ett alternativ till en levertransplantation. Detta är därför ett nytt och mycket lovande forskningsområde, men tyvärr finns det fortfarande ett flertal hinder och svårlösliga problem i samband med denna typ av behandlingsmetod som gör den opraktisk att använda i kliniken.

Syfte: Den aktuella avhandlingen är inriktad på att validera och utvärdera olika typer av cellers förmåga att transplanteras, och hur dessa kan anpassa sig till den nya miljön i en leverskadad djurmodell. På sikt kan denna typen av forskning leda till förbättrade och säkrare behandlingar för leversjuka.

Material och metoder: En liten andel av de totala leverceller som kommer ifrån foster är kolonibildande och har en mycket stor celldelningspotential. Således kan användningen av dessa fosterleverceller (hFLC) vara en lämplig kandidat för cellterapi och diagnostik. Vi har utvärderat humana fosterlevercellers tillväxt under cellodling, samt undersökt dess potential för cellterapi till en leversjuk mus med nedsatt immunförsvar (nakenmus).

Resultat och diskussion: Den aktuella avhandlingen visar att förbehandling med cytokiner förbättrar cellernas förmåga att integrera med värddjuret efter transplantation, samt att när en kombinerad cellterapi genomförs med hFLC-celler och med en annan sorts stamceller (mesenkymala stamceller), så ökar sannorlikheten för en bättre cellterapi behandling. Avhandlingen beskriver även ett effektivt protokoll att isolera leverstellatitceller från både foster och färdigutvecklad lever, samt beskriver att när dessa manipuleras så att cellernas livslängd ökar, leder detta till en förbättrad tillväxt inför cellterapiapplikationer vilket har en stor betydelse för nya behandlingar av leverfibrosis.

Sammanfattning: Denna avhandling beskriver nya förbättrade och säkrare strategier för levercellstransplantationsappliaktioner än vad som tidigare har bevittnats. Resultaten har stor betydelse för att effektivisera och öka behandlingsmöjligheter för leversjuka.

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ABBREVIATIONS

Abbreviations

- AFP - Alpha-fetoprotein
- ALB - Albumin
- ALF - Acute liver failure
- ACLF - Acute-on chronic liver failure
- BSA - Bovine serum albumin
- CNS - Crigler–Najjar syndrome
- c-Met - Met proto-oncogene
- CoTx - Co-transplantation
- C_t - Threshold cycle
- D-gal - D-galactosamine
- DAPI - 4', 6-diamidino-2-phenylindole
- EBM - Experimental biomedicine
- ELISA - Enzyme linked immunosorbent assay
- FHTx - Fetal hepatocyte transplantation
- FISH - Fluorescent *in situ* hybridization
- G-6-Pase - Glucose-6-phosphatase
- hFHs - Human fetal hepatocytes
- hFLCs - Human fetal liver cells
- hFLMSCs - Human fetal liver mesenchymal stem cells
- HLPCs - Human liver progenitor cells
- HSCs - Hepatic stellate cells
- hTERT - human telomerase reverse transcriptase
- ICC - Immunocytochemistry
- IHC - Immunohistochemistry
- iPSCs - Induced pluripotent stem cells
- LCT - Liver cell transplantation
- MACS - Magnetic-activated cell sorting
- MMPs - Matrix metalloproteinases
- MSCs - Mesenchymal stem cells
- MTx - Mesenchymal stem cells transplantation.
- NASH - Nonalcoholic steatohepatitis
- OLTx - Orthotopic liver transplantation
- PH - Partial hepatectomy
- qPCR - Quantitative polymerase chain reaction

- SECs - Sinusoidal endothelial cells
- SOD - Superoxide dismutase
- SV40-LT - Simian virus 40 large T antigen
- TIMP - Tissue inhibitor of metalloproteinase
- UCD - Urea cycle disorder
- WB - Western blotting

INTRODUCTION

Introduction

According to WHO, 29 million people in Europe are affected by a chronic liver disease and 170,000 die yearly from liver cirrhosis, accounting for 1.8% of all deaths in Europe (Blachier, Leleu et al. 2013). Globally, 150 million people are chronically infected with hepatitis C virus alone, resulting in 350,000 deaths each year (WHO 2013). Although promising treatments for hepatitis infections exist, the vast majority of liver-insults prevails and progress to liver failure.

The liver was the second visceral organ to be allotransplanted in humans. In 1963, an American physician Thomas Starzl (father of modern transplantation) performed the first human liver transplantation in a patient with biliary atresia (McClusky, Skandalakis et al. 1997). Since then, liver transplantation with different approaches (Starzl 2012) has offered life saving opportunities for many patients each year. However, because of increased liver disease morbidity and mortality (globally), orthotopic liver transplantation (OLTx) is not a sufficient alternative due to the lack of healthy organ donors and related hurdles before and after transplantation. New innovative treatments expand the donor pool through live organ donation and split liver transplantation, the latter allowing transplantation of two recipients from one donor organ (Broelsch, Emond et al. 1988) and a decreased waiting list by recruiting live liver donors. Despite these efforts, organ shortage continues to be a major problem. Therefore, liver cell therapy (LCT) has been suggested; an application that is gaining recognition to serve as a bridge or an alternative to orthotopic liver transplantation for patients with acute liver failure (ALF), acute-on chronic liver failure (ACLF) or

genetic defects. Ideally, human hepatocytes should be produced and expanded in laboratories for offering a first line of curing treatment, but unfortunately still seen only as an interim palliative treatment option. To make LCT work requires multiple cell infusions over an extended period of time with varying doses of cell number. Furthermore, the increased number of non-reproducible protocols available for LCT act as hurdles in translating this technique to the clinic. To overcome these obstacles, the approach for LCT has to be refined through thorough evaluation and to be improved with respect to its long-term functional efficiency.

Liver anatomy

The liver is the largest internal glandular organ of the body weighing about 2-2.5% of the body weight (Average: 1.5 kg in an adult human). Liver secretions play an important role in the metabolism of food. The liver receives its blood supply from the portal vein and the hepatic artery. The portal vein carries deoxygenated blood (rich in nutrients and bacterial toxins absorbed from the intestine) from the pancreas, spleen, stomach, small intestine and large intestine, and supplies around 75-80% of the hepatic blood flow (Schenk, Mc et al. 1962, Rappaport 1980, Vollmar and Menger 2009).

The liver is anatomically divided into a right and a left lobe, however, according to Couinaud's classification (1957), it can be divided into eight functionally independent segments (Couinaud 1957) with virtual lines dividing parenchyma (Fig. 1). Knowledge of the anatomical landmarks has made it possible to surgically divide the liver into separate functional parts through the split liver

procedure (Broelsch, Emond et al. 1988). The anatomical landmarks are also used in planning a liver resection.

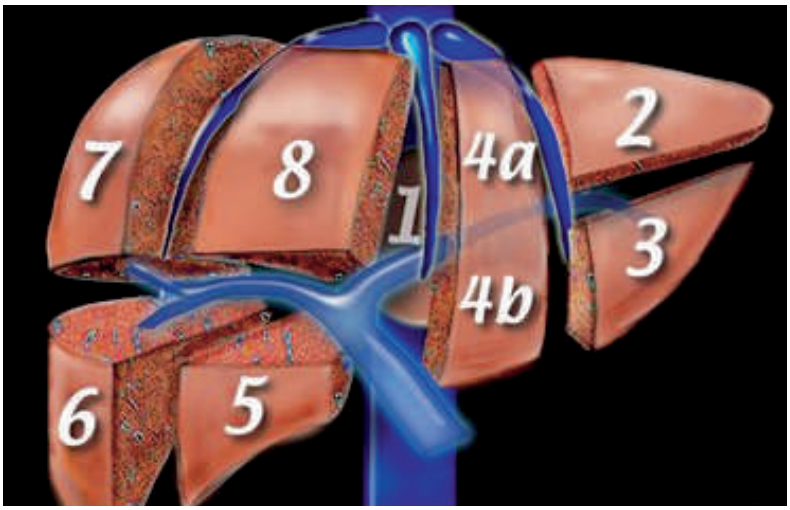

Figure 1. Segmental anatomy of human liver (adapted from (Smithuis 2006))

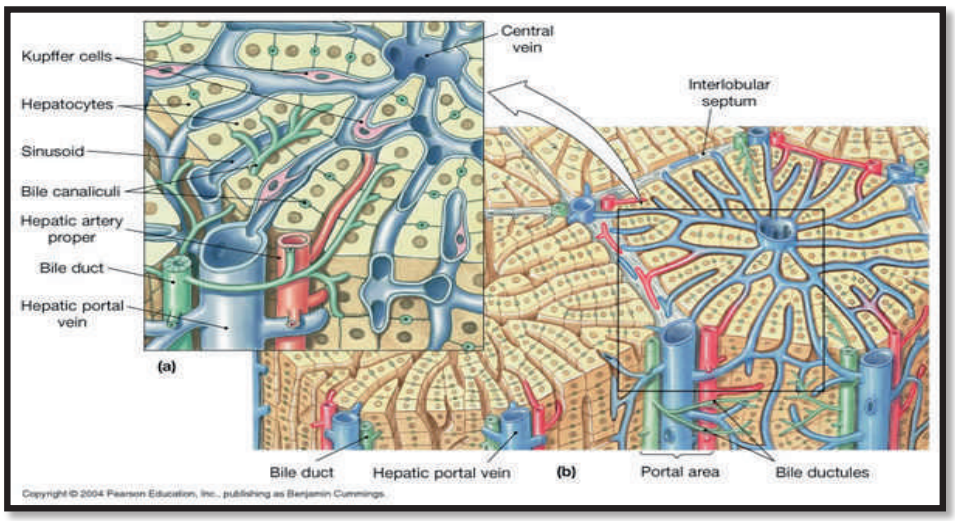

Figure 2. Functional unit of liver - Hepatic lobule

Each liver lobe is divided by connective tissue into approximately 0.1 million liver lobules that are the basic functional unit of the liver (Fig. 2). The liver cells (hepatocytes) comprise about 60-80% of the liver (Fig. 3). They are polygonal and approximately 30 μm in diameter. The nucleus of the hepatocyte is single/multiple and cells divide by mitosis. The lifespan of human liver cells is proposed to be 150-200 days (Dooley 2011) and of rats 191-453 days, but this information lacks reliability (Kuntz and Kuntz 2005).

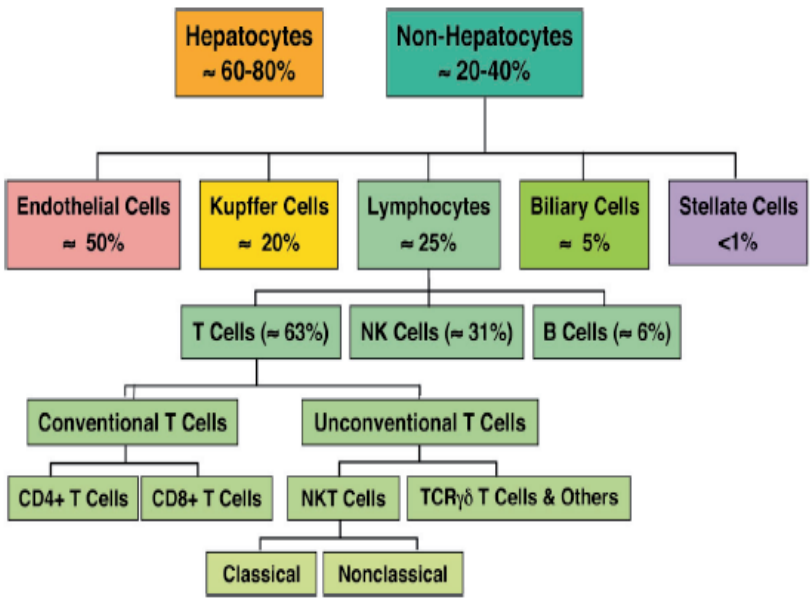

Figure 3. Percentile distribution of human liver cells [adapted from (Racanelli and Rehermann 2006)]

The fluid-filled space of Disse separates the hepatocytes from the sinusoidal endothelial cells (SECs), which are special because they lack intercellular junctions. Instead, they create fenestrae in-between the endothelial cells,

thereby allowing contact between the circulating blood and the hepatocytes (Braet, Riches et al. 2009). The hepatic stellate cells (HSCs) are found within the space of Disse. Their key functions are the production of extracellular matrix (ECM) and the storage of vitamin A. The stimulation/activation of HSCs is a cellular event in liver regeneration and liver fibrosis (Friedman 2008).

The hepatocyte has three surfaces: 1st facing the sinusoid and space of Disse, the 2nd facing the canaliculus and the 3rd facing neighboring hepatocytes. They lack basement membrane. The sinusoids are lined by endothelial cells. Phagocytic cells of the reticulo-endothelial system (Kupffer cells) are associated with the sinusoids, and the HSCs, which have also been called fat storing cells/ Ito cells/ lipocytes (Dooley 2011).

Development of the fetal liver

The fetal liver is a rich source of precursor cells, which are believed to be multipotent stem cells with a high proliferative ability. Human fetal liver appears from the foregut endoderm after 4 weeks of gestation and develops rapidly, such that bile is produced by 14 weeks. At the 1st trimester, hematopoiesis starts from the liver instead of the yolk sac (Migliaccio, Migliaccio et al. 1986), and contains both hepatic and hematopoietic progenitors. Hepatic cells express hepatocyte markers [e.g., albumin (ALB), alpha-fetoprotein (AFP), α-1 microglobulin, glycogen, glucose-6-phosphatase (G-6-Pase) and Hep-Par-1], and biliary markers e.g. gamma-glutamyl transpetidase (GGT), dipeptidyl peptidase IV (DPPIV), CK-19 and Das-1-monoclonal antibody-reactive antigen (Haruna, Saito et al. 1996, Badve, Logdberg et al. 2000). Human hepatoblast

(present in large numbers at fetal liver) express all these markers and under culture conditions, progenitor cells proliferate for several months (Malhi, Irani et al. 2002). Since, hFLCs are a reliable and safe cell source and have a high proliferative capacity, we explored and evaluated new cell therapy approaches using hFLCs on mouse models of liver injury, which is further described in this thesis.

The magnitude of liver disease

The liver has a high capacity for regeneration *in vivo*, which facilitates thorough restoration of liver architecture and the re-establishment of its specific functions after various types of liver injury (Palmes and Spiegel 2004). This is being explored in the tissue-engineering field. Despite improved preoperative evaluation, surgical methods and thorough perioperative care, some patients still experience postoperative functional liver failure with insufficient regeneration known as small for size syndrome (SFSS) (Mortensen and Revhaug 2011).

In a wider perspective, liver cancer has the 5th highest cancer incidence in the world, and is the 3rd highest cause of cancer related deaths (Parkin, Bray et al. 2001), with resection of the liver remaining the only curative option (Kanat, Gewirtz et al. 2012). The main causes of cirrhosis globally are hepatitis B and C and alcohol abuse. Changing patterns of alcohol consumption and the increasing incidence of obesity and diabetes suggest that the burden of fibrosis and cirrhosis related to alcohol and nonalcoholic steatohepatitis (NASH) will continue to increase (Fallowfield and Iredale 2004). Due to numerous hurdles

on the way to a possible transplantation, all patients do not get an equal chance of survival. This demonstrates the demands for new improved treatment alternatives.

Medical treatment of liver failure

There are more than 100 different liver diseases, and for the vast majority there is no specific medical therapy available. For diseases caused by alcohol abuse or obesity, prevention is the best option. Patients with hepatitis B or C can receive antiviral medication with increased efficiency if the disease is detected and treated on time. However, patients with metabolic diseases often cannot be treated and the only option remaining is the replacement of the native liver function through OLTx (Tavill 2009).

Surgical treatment for liver failure

Since last century, advances in the surgical field have improved the quality of life for liver failure patients, with techniques such as liver resection and orthotopic liver transplantation. Currently, for patients with malignancies, the first option is to surgically remove the tumor using liver resection techniques. Hepatic resections are both associated with, and dependent on a rapid proliferation and regeneration of the remnant liver. However, liver failure following partial hepatectomy still occurs, mainly due to a massive resection, a pre-existing liver disease (neoadjuvant chemotherapy) or advancing age (Helling 2006).

OLTx was introduced as a treatment option for patients with liver failure in the mid-sixties (Starzl 2012). The method saves the lives of thousands of patients every year, with excellent long-term results. Since the OLTx procedure requires an organ from live/deceased donor, only a limited number of patients can be saved this way due to the scarcity of donor organs *vs* increased morbidity. As mentioned earlier, innovative techniques have expanded the donor pool by splitting the donor liver for two recipients and the use of live organ donation (Starzl 2012). However, due to the vast number of patients, these techniques will not be enough to solve all the problem for patients in need of liver replacement therapy. Although surgical, medical and diagnostic techniques for liver failure have progressed, mortality while still on the waiting list has increased tremendously, and is now up to 40% over the last few years due to lack of donors and increased patient numbers (Prakoso, Verran et al. 2010, Yu, Fisher et al. 2012).

Liver cell transplantation (LCT) truth *vs* hype

Hepatocyte transplantation has been attempted to cure metabolic liver disorders and end-stage liver diseases. However, the assessment of its effectiveness is complicated by the shortage of human hepatocytes and difficulties in their expansion and cryopreservation. Current advances in cell biology have led to the notion of “regenerative medicine”, which is derived from the increased evidence of the therapeutic potential of stem cells as a viable and on-shelf option. There are different types of SCs that are theoretically eligible for liver cell replacement. These include embryonic and fetal SCs, induced

pluripotent cells (iPSCs), endogenous liver SCs, and extrahepatic adult SCs (Allen and Bhatia 2002).

LCT is an innovative technique that is especially promising for treating children because it is less invasive than OLTx. The two key indications for LCT are ALF and hepatic-based inborn errors of metabolism e.g. Crigler–Najjar syndrome (CNS) type 1, familial hypercholesterolemia, glycogen storage disease type 1, α-1-antitrypsin deficiency, infantile Refsum's disease, progressive familial intrahepatic cholestasis type 2, and urea cycle disorders (UCD) (Meyburg, Schmidt et al. 2009).

LCT has some exceptional advantages compared to whole or partial liver transplantation. LCT is not only a less invasive technique, but also a promising on-shelf solution to a shortage of healthy liver donors either by utilizing cells isolated from resected liver or aborted fetal liver or discarded liver from the transplantation unit. In addition, the LCT procedure can be repeated several times during the clinical course, without disturbing the patient's quality of life. All of these issues make this technique, especially appealing in pediatric patients. Due to its novelty, however, only a few patients have been treated with this technique so far.

Adult hepatocyte transplantation is emerging as an alternative interim support (bridge to OLTx), for patients waiting for a donor organ (Najimi and Sokal 2005, Stephenne, Najimi et al. 2006). Despite reported improvements in these patients, significant problems appeared due to (a) inefficient engraftment, (b) death or ectopic distribution of cells that did not engraft in the target tissue, (c)

emboli formation, (d) immunological rejection, and (e) transient effects of transplanted cells (Dalgetty, Medine et al. 2009, Bonora-Centelles, Donato et al. 2010, Ribes-Koninckx, Ibars et al. 2012).

Liver regeneration

In liver regeneration studies, animal liver failure models and liver cell therapy are closely interconnected fields, which have been developing since 18th century. The term partial hepatectomy (PH) means surgical resection of one or more liver segments. The term 'hepatectomy' is derived from the Greek words (*hepat*: "liver" and *ectomy*: "to cut out"). In the last several years, liver regeneration research has evolved in several directions (from the extrinsic hepatic growth factors, to the intrinsic changes in the ECM, the intracellular signal transduction mechanisms and the genetic response in the liver) and different theories (flow rate, oxygen tension, humoral content) have been updated and revised (Mortensen and Revhaug 2011).

According to modern concepts of liver regeneration, the liver has three levels of cells that can respond to liver injury and loss of hepatocytes (Sell 2001). First, the numerous mature hepatocytes respond to mild liver injury within 1 to 2 cell cycles. Second, the less numerous intra-organ ductal progenitor cells respond by prolonged, but limited proliferation. Third, stem cells entering from the circulation begin to participate in the liver regeneration (Petersen, Bowen et al. 1999). In this latter mode, bone marrow cells enter first as an intermediate cell population in response to injury, which then mature into hepatocytes (Levicar, Dimarakis et al. 2007).

Liver regeneration after partial hepatectomy is one of the most studied models of cell, organ, and tissue regeneration. The intricacy of the signaling pathways initiating and terminating the process has provided paradigms for regenerative medicine. The process of liver regeneration is divided into three phases: initiation, proliferation and termination. During liver regeneration two distinct pathways are activated - the growth factor and the cytokine-regulated pathway. These pathways have checkpoints that could be feedback-inhibited, thus regulating liver growth and size (Koniaris, McKillop et al. 2003). A variety of animal models have been created and used for evaluating liver regeneration and LCT of liver injury (Rahman and Hodgson 2000, Palmes and Spiegel 2004, Meyburg, Schmidt et al. 2009). There are a number of events and parameters that have been discovered, which help to design and plan LCT experiments judiciously.

Although some mechanisms regulating hepatocyte proliferation and apoptosis have been established (Kataoka, Nakai et al. 1994, Togo, Makino et al. 2004, Nakashima, Inui et al. 2006, Apte, Thompson et al. 2008, Hsieh, Chen et al. 2009, Weng, Zhuang et al. 2014), clinical trials failed to capitalize on the LCT technique from lab bench to bedside. Few interesting aspects about liver are hepatostat (a self governing mechanism, by which *in vivo* liver lobe growth is controlled in a resected liver) (Nygård 2013), parenchymal shift (one low is ischemic, other lobe gets hypertrophied) (Rous and Larimore 1920), natural regeneration (hepatocytes re-enter the cell cycle).

Overview on Liver Cell Therapy (LCT)

In response to the increasing incidence of liver disease and the relative shortage of donor organs, many investigators have developed cellular therapies using isolated hepatocytes. Such approaches must consider both the source of hepatocytes and crucial stabilization of liver-specific function. Cell-based therapies that are considered for LCT can be generally classified as extracorporeal devices, cell transplantation and tissue-engineered constructs (Galun and Axelrod 2002, Vindigni, Cortivo et al. 2009).

Although advances in stem cell biology and the discovery of iPSCs have made the prospect of cell therapy and tissue regeneration possible, hurdles in generating the cells, maintaining their functionality and controlling the non-cancer state have kept clinicians waiting. Cell therapies embrace great assurance to repair, restore, replace or regenerate affected organs and may perform better than any pharmacological or mechanical device. There is an abundant amount of evidence supporting the contribution of adult stem cells, in particular those of bone marrow origin, to regenerate the liver and pancreatic islet cells (Levicar, Dimarakis et al. 2007). Despite their potential, very limited work has thus far performed on human fetal hepatocytes (hFHs) due to ethical constraints in many countries.

Hepatocyte transplantation has been attempted to cure ALF, metabolic errors, ACLF and end-stage liver diseases. As mentioned earlier, the assessment of its efficacy is complicated by the shortage of human hepatocytes and difficulties in their expansion or cryopreservation (Allen and Bhatia 2002). In brief, the lack of

healthy liver donors; the absence of a standard protocol for the isolation and expansion of cells (variation of isolated cell number occurs using same protocol in different livers, and variations in producing large populations without losing its functions); ethical/religious issues; lack of expertise, an ideal translating research (animal experiment do not necessarily predict human complication or requirement) or facilities (cost and expert biologists needed), all result in LCT being classed as mission impossible for many clinicians.

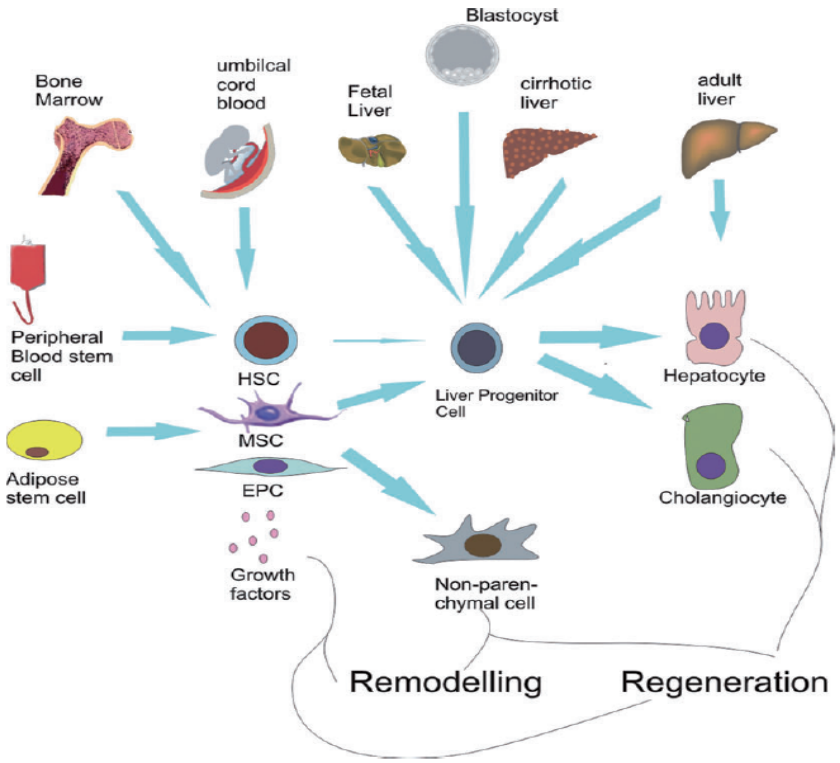

Figure 4. Cell sources used for LCT (adapted from (Dan and Yeoh 2008))

Cell-based approaches rely on a variety of cell sources as mentioned above (Dan and Yeoh 2008)(Fig. 4), whereas the safety and availability vary which decides

its use. Primary cells or stem cells after transplantation interact with the microenvironment of liver to provide key liver-specific functions. A fundamental understanding of these clues helps in designing the next generation of extracorporeal devices, cell transplantation therapies and tissue-engineered constructs (Allen and Bhatia 2002).

Furthermore, strategies to harness and regulate host liver regeneration could even offer the potential to reverse chronic liver fibrosis and cirrhosis, currently thought to be irreversible. In addition, immunological issues will be an important consideration for cell-based therapies; therefore, contributions from transplantation immunology that aim to promote graft tolerance are of great interest (Knechtle 2000, Starzl 2012). Finally, development of predictive animal models to evaluate liver therapies will offer a vital preclinical assessment of new therapies as they emerge.

The potential candidate cells, which have been used, include autologous primary cells, cell lines and several stem cell types, including bone marrow stem cells, cord blood stem cells and embryonic stem cells (Fodor 2003). This thesis will focus on an additional source of cells (hFLCs) for LCT.

In recent years, advances in stem cell biology, including embryonic and somatic stem cells have made tissue regeneration a potential clinical reality, and several experimental studies have shown great promise for stem cells as successful therapies (Assmus, Schachinger et al. 2002, Wollert, Meyer et al. 2004). Limited clinical trials completed (Yu, Fisher et al. 2012, NLM and NIH. 2014) limits the possibility of LCT reaching to a larger population over few more years. Despite

the unquestioned totipotency (total potential in one cell) of embryonic stem cells, there are numerous unanswered biological questions about the regulation of their growth and their differentiation. For example, earlier trials evaluating the safety profile of unselected embryonic stem cells for transplantation in early studies demonstrated dysregulated cell growth that resulted in teratoma formation (Reubinoff, Pera et al. 2000).

The main role of adult stem cells, which make up approximately 1–2% of the total cell population within a specific tissue, is to replenish the tissue's functional cells in appropriate proportions and numbers in response to 'wear and tear' loss or direct organ damage (Fang, Min et al. 2004). They are vital in the maintenance of tissue homeostasis by continuously contributing to tissue regeneration and replacing cell loss during apoptosis or direct injury (Li and Xie 2005). Adult stem and progenitor cells are capable of self-renewal and differentiation into one or more mature cell types. They are able to maintain their populations within the human body through asymmetric and symmetric divisions to create differentiated and undifferentiated progeny (Preston, Alison et al. 2003). These properties make hFLCs an ideal candidate for stem cell-based therapies and tissue engineering, irrespective of their *pros* and *cons*.

Cell transplantation is typically performed by intravenous or peritoneal administration of hepatocytes in suspension. This mode of cellular therapy exploits a key advantage of the *in vivo* hepatic microenvironment: adult hepatocytes engrafted in the liver through the spleen can proliferate extensively and reconstitute liver function. In a mouse model of cell transplantation, a

single hepatocyte was calculated to have the potential to go through 34 population doublings, or give rise to 1.7 x 10^{10} cells (Rhim, Sandgren et al. 1994, Overturf, al-Dhalimy et al. 1997). As above, various animal models have repeatedly proven the success in the LCT procedure, hepatocyte engraftment and proliferation (Brezillon, Kremsdorf et al. 2008). However, similar approaches have limited success in humans due to variations in disease or immune status of individuals, risk of cancer and active and complex immune system (Yu, Fisher et al. 2012, Lee, Tang et al. 2013).

Critical elements for an effective regeneration are the hepatotropic environment and the available sites for cell growth. In animals, partial hepatectomy, portocaval shunting, transgenic injury, or administration of hepatotoxins before cell transplantation typically generates this stimulation. Delivery of the isolated cells has been attempted by injection into peripheral veins, or the portal vein, or the splenic artery, or the peritoneum or the spleen (Jameson 2008). The inefficient engraftment of hepatocytes and the limited cell survival after transplantation remain major limitations of these techniques (Joseph, Malhi et al. 2002, Sosef, De Bondt et al. 2007, Malato, Naqvi et al. 2011). Earlier studies demonstrated problems with linking animal survival and the function of transplanted cells, either due to the unexpectedly high animal survival form relatively few injected cells, or due to the beneficial effects from non-viable hepatocytes or non-hepatocytes (Makowka, Rotstein et al. 1980, Yarmush, Dunn et al. 1992). These varied results, added confusion to the therapeutic strategy and demanded further analysis on the selection of study model and design for LCT applications. Transplanted hepatocytes also require time to

engraft and grow (doubling time in mice, 28 h) (Weglarz, Degen et al. 2000) and this may limit the utility of the procedure for certain clinical indications. Furthermore an inadequate hepatocyte supply is another barrier. For example, metabolic defects have been replaced with a relatively small liver mass compared to the requirement for ALF. The normal adult liver contains approximately 10^{11} hepatocytes; therefore, 2% of hepatocytes would require 2 billion engrafted cells (Allen and Bhatia 2002). To date, there is no standardized formula to guess the exact dose of cell number required for a successful treatment in a given case because of variation in stage of liver failure, variation in immune/inflammatory status.

Currently, a limited supply of allogeneic human hepatocytes is obtained by collagenase perfusion of organs, which is inappropriate for transplantation (Hewitt, Corno et al. 1997). Studies have also highlighted that the limited lifespan of adult hepatocytes may contribute to the development of cirrhosis (Rudolph, Chang et al. 2000). Therefore, telomerase expression in differentiated human hepatocytes may extend the lifespan of transplanted cells *in vivo*. Cell transplantation clearly holds promise as a therapy for a subset of clinical hepatic syndromes, but faces the same challenges with cell sourcing, inadequate function in later passages, and safety (as for other *in vivo* methods such as tissue-engineered constructs). The parameters involved in LCT output are numerous and a complex web, which demands a judicious logical selection procedure.

It is estimated that for treating various metabolic liver disorders, approximately 5% hepatocyte replacement by transplanted cells is required (Ribes-Koninckx, Ibars et al. 2012), however, failure to engraft enough number of viable-functional-transplanted cells (Wan, Zhang et al. 2013), remained unsolved mystery. There are two main obstacles that could lead to low donor cell mass in the recipient. First, the vast majority of donor hepatocytes is cleared from the liver parenchyma during the engraftment process (Gupta, Rajvanshi et al. 1999), secondly, only limited proliferation (Shen, Zheng et al. 2008, Song, Sharma et al. 2010) (lack of expandability) of surviving donor cells is observed in the host liver. Thus, there is a need for designing strategies that could select the specific sources and methods (tailor-made as per patients need). This shall amplify the engraftment and proliferation of transplanted cells, and transform LCT from being a temporary solution (OLTx-bridge) to become a curing remedy.

The severe scarcity of healthy donor liver hampers the availability of hepatocytes for transplantation. Moreover, the amount of donor cells that can be safely infused into the portal circulation during a single procedure is particularly low, usually not more than 5% of the liver mass (Ribes-Koninckx, Ibars et al. 2012). Typically, hepatocyte transplantation just partially corrects the metabolic disorder. Low initial engraftment and subsequent limited proliferation contributed to the inadequate donor cell material in the recipient, and may lead to ineffective therapies (Wan, Zhang et al. 2013). The majority of patients that received LCT eventually also received an OLTx, which shows that LCT remained only as a bridge to transplantation.

Cell engraftment in the liver involves entrapment of transplanted cells in hepatic sinusoids, migration into the space of Disse and integration into the liver parenchyma. A series of engraftment-associated events such as activation of liver non-parenchymal cells, release of inflammatory mediators and hepatic ischemia–reperfusion injury produce both adverse and beneficial effects upon the cellular graft. For example, activated Kupffer cells clear a large fraction of donor cells from portal spaces within 24 hours post-transplant. On the other hand, Kupffer cells release adhesion molecules, such as intercellular or vascular cell adhesion molecules, which promote hepatocyte attachment to the sinusoidal endothelium. The multistep process takes more than a week before transplanted hepatocytes can completely reconstitute plasma membrane structures (including gap junctions and bile canaliculi) and start to function like normal endogenous hepatocytes. It is observed that no more than 30% of transplanted hepatocytes engraft successfully in the recipient liver (Guha, Ghosh et al. 2000, Wan, Zhang et al. 2013).

Subsequently, preferential proliferation of engrafted hepatocytes requires two essential conditions: liver injury or liver parenchymal loss to supply a proliferative stimulus to hepatocytes, and inhibition of mitotic division of endogenous hepatocytes; both will provide an advantage to engrafted cells to proliferate. The latter point is valid only for cancer patients treated with LCT, or in cases of experimental animal studies regulating proliferation to the engrafted cells only. Other approaches (Wan, Zhang et al. 2013) that may also be beneficial are: (a) increasing the sinusoidal spaces, (b) regulation of the sinusoidal endothelial cell barrier, (c) controlling the inflammatory reaction, (d)

preferential proliferation of the transplanted cells, (e) liver-directed irradiation and (f) reversible partial portal vein embolization.

It has been found that most animal experiments achieved enhanced cell proliferation by preconditioning of the host liver. Although, a number of preconditioning regimens (e.g. c-Met, irradiation or chemicals) have been tried, they were discontinued due to various side-effects (e.g. senescence, radiation hazards or cancer) on LCT recipient (Kaldenbach, Giebeler et al. 2012, Serra, Marongiu et al. 2012).

In certain models, nearly a complete repopulation with transplanted cells were seen (Grompe 1999). Unfortunately, the harsh methods used for preconditioning in these animal studies can hardly be transferred to human applications. However, techniques that are regarded less effective in animal models like low-dose irradiation or selective portal venous ischemia could theoretically be adapted to human LCT (Stephenne, Najimi et al. 2005) in a few selected clinical cases, but warrants long-term studies reveal potential drawbacks or side effects.

It is difficult to quantify the cell transplantation success, and the amount and duration of the therapeutic benefits are also difficult to assess. In pancreatic cell transplantation studies, there was a continuous decline of enzyme activity observed during the first five years after pancreatic islet cell transplantation for type 1 diabetes, indicating a slow loss of transplanted cell's functions. Although such patients usually have pre-formed antibodies against islet cells, similar effects may appear after LCT (Meyburg, Schmidt et al. 2009). The longest

reported duration of beneficial effects following a LCT is 36 months, and was reported in a 47 years old woman with glycogen storage disease (also known as glycogenolysis or dextrinosis) (Muraca, Gerunda et al. 2002). This is an exceptional case, as the mean duration of observed positive effects in children with metabolic disorders is 9.7 months. However, most of the reported children were bridged to transplantation (Meyburg, Schmidt et al. 2009). Therefore, it cannot be judged whether long-term stabilization or even a cure of the disease by LCT would have been possible. For each patient, the benefit of increased metabolic stability has to be balanced against possible serious side effects of the immunosuppression. Particularly in UCD patients, where every infection could lead to protein catabolism and serious hyperammonemic crisis, increased susceptibility to infections could be fatal. In a recently published animal model, tolerance without the need of immunosuppression could be achieved by genetic modification of the transplanted hepatocytes (Mashalova, Guha et al. 2007), but this concept is still quite far from today's clinical reality due to its potential risk of tumor formation.

In contrast to the excellent results of numerous animal studies, critical questions still remain in human LCT. In children with ALF, LCT seems favorable in comparison to artificial or bioartificial liver support systems to safely bridge patients for OLTx. In ACLF, LCT is not a realistic option because of elevated portal vein pressure and fibrotic/cirrhotic changes in the liver. The same holds true for metabolic diseases with structural damage to the liver, like progressive familial intrahepatic cholestasis or alpha-1-antitrypsin deficiency (Meyburg, Schmidt et al. 2009).

In this thesis, we provide an evidence for *in vitro* cultured fetal liver cells as an important reliable cell source for LCT and their *in vivo* variations in engraftment numbers, pattern and cellular behavior of hFLCs individually or together with regimens used in respective studies.

AIMS OF THE THESIS

Aims of the thesis

The overall aim of this thesis is to define different approaches in enhancing cell engraftment using a mouse model of liver failure. The specific objectives are:

- **To** test whether mesenchymal stem cells can have a facilitating effect during hepatocyte cell transplantation.
- **To** test whether SV40 transduced hFLCs maintain phenotypic and *in vivo* functionality over several passages.
- **To** study whether temporarily priming of the liver parenchyma with selective chemokine ligands enhance the engraftment of transplanted fetal hepatocytes.
- **To** study whether CD271 antigen in hFLCs can be a marker for HSCs, portal fibroblasts and hepatic vascular smooth muscle cells.

MATERIALS & METHODS

Materials and methods

The overall study design is as follows (for all papers included in this thesis):

In vitro evaluation	• Cell isolation – aborted human fetuses • Cell population purification – FACS and MACS • Cell culturing, genetic profiling and transduction • Cell migration assay • Cell functionality assay – Urea, Ammonia and SOD • Cytokine analysis • Matrix metalloproteinase analysis • Biochemical staining – G-6-Pase, glycogen • Cell characterization using ICC & FACS- Cancer, Hepatic & MSCs markers (MACS – Magnetic activated cell sorting, SOD – Superoxide dismutase)
In vivo evaluation	• Preparation of chemically induced liver injury in a nude mouse model • Partial hepatectomy • Preconditioning regimen and intrasplenic cell transplantation • Quantification of human ALB in mice sera - ELISA • Quantification of c-Met in tissue - ELISA • Quantification of engrafted cells – IHC • WB and qPCR for human specific protein and mRNA detection

The main methods used in this thesis are shortly described below. More detailed descriptions are found in the 'Materials and methods' sections of respective papers (I-IV).

Human liver tissue samples (Paper I-IV)

The human liver cells were isolated from aborted fetuses (fetal liver progenitor cells) in gestation weeks 7-10 (Fig. 5) or adult hepatocytes from healthy organ donors. All protocols used in the present thesis were approved by the Swedish Institutional Review Board. Adult liver tissue weighing app. 70-100 g from different deceased healthy organ donors was obtained after informed consent. A biopsy piece of 2 cm^3 from each adult liver was snap frozen in liquid nitrogen and stored at -80°C, which was used at a later time point for respective analysis.

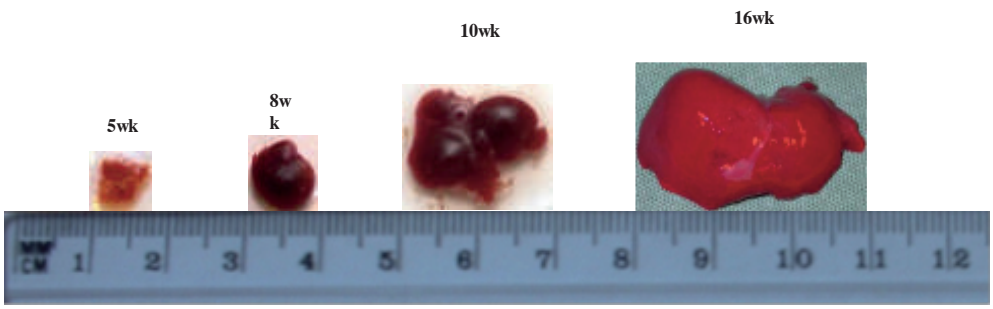

Figure 5: Size and shape of the developing human fetal liver at various gestation times (Bhattacharya and Stubblefield 2013).

Liver cells were isolated from adult human livers using the standardized two-step collagenase perfusion method (Berry and Friend 1969, Seglen 1976, Strom SC 1998). A single-cell suspension was prepared from human fetal livers as described elsewhere (Nowak, Ericzon et al. 2005, Begum, Joshi et al. 2010, Joshi, P et al. 2012).

Culturing of hFLCs (Paper I-IV)

All fetal tissue samples were obtained from donors that were serologically screened negative for syphilis, toxoplasmosis, rubella, HIV-1, cytomegalovirus, hepatitis B and C, parvovirus and herpes simplex types 1 and 2. Primary hFLCs were obtained by homogenizing liver lobes against a 100 μm mesh. Obtained cells were cultured and processed further for transformation or isolation protocols as mentioned in the respective studies (Paper I-IV).

The various primary cells and cells lines used in the four studies were cultured in cell specific media as follows: hepatocytes were cultured in a Dulbecco's Modified Eagle's Medium (DMEM) with supplements (details are in Paper I), MSCs were cultured in MesenCult®™ medium with further supplements (details are in Paper I) and $CD271^{+/-}$ cells were cultured in stellate cell medium (details are in Paper IV). Freshly isolated cells, or cells with an earlier passage number were used for all analysis.

Isolation of a cell population using magnetic beads (Paper I-IV)

MACS is a method for separation of various cell populations depending on their surface antigens (CD molecules). MACS is a commonly used method in cancer

research, immunology, neuroscience, and stem cell research. This method is reliable and reproducible for isolating cells of interest from mixed cell populations.

The hFLCs were selected using a positive selection kit with magnetic nanoparticles (Stem Cell Technologies, Vancouver, Canada), according to the manufacturer's instructions. Three magnetic separations, 5 minutes each, were carried out using an EasySep magnet (Stem Cell Technologies, Vancouver, Canada). Later cell fractions were plated into non-coated T75 culture flasks. The culture medium was changed every third day until the cells reached 60-70% confluence. The cells were then trypsinized with Trypsin-EDTA (Invitrogen) and further cultured after splitting them 1:4.

Isolation of a cell population using cell sorting (Paper IV)

In cell biology, flow cytometry is a laser-based biophysical technology employed for cell counting, cell sorting, biomarker detection and for protein engineering. It works by suspending the cells of interest in a stream of fluid, which is then measured by an electronic detection apparatus. We have used this technique due to its possibility of using multiple parameters to isolate a specific cell population with high reliability and reproducibility.

In paper-IV, CD271 positive cells were isolated using a FACS Aria II (Becton and Dickinson, USA) from adult and fetal human livers. Negative controls included non-stained cells and cells stained with the control APC mouse IgG1 isotype. Compensation beads (Becton Dickinson) were used to compensate

fluorochrome spectral overlap. Finally, cells were sorted using the FACS Aria II. After exclusion of cell debris and cell doublets by applying a forward *vs* side-scatter gate, a second gate was also applied to sort the specific cell population.

Transduction of hFLCs (Paper I-III)

In paper I (CoTx of hFLCs and hFLMSCs), the hFLMSCs were transduced using lentiviral transfer vector virions (pHR'EF1 α GFPSIN). The hFLCs were transduced using the CMV/SV40LT/PAC plasmid for paper II (SV40-LT transduced hFLCs), and the pCDM7/EF1α/hTERT/PAC plasmid for Paper III (hTERT transduced hFLCs and chemokines). Already transduced stable cell lines were obtained from NovaHep AB and used for respective studies.

Cell migration assay (Paper III)

Cell migration is a central process during development, and for the homeostasis of multicellular organisms. Tissue formation during embryonic development, wound healing or immune responses all require an orchestrated movement of cells in particular directions to specific locations. Errors during this process have serious consequences, including intellectual disability, vascular disease, tumor formation and metastasis. An understanding of the mechanism, by which cells migrate may lead to the development of novel therapeutic strategies for controlling invasive tumor cells or cell engraftment for example, evaluated in this thesis. Cells often migrate in response to specific external signals, including chemical and mechanical signals.

Recombinant human chemokine ligands from the CXCL family (all from R&D systems, Oxon, UK)(Nava, Westgren et al. 2005, Nowak, Ericzon et al. 2005, Begum, Joshi et al. 2010) were used to study chemotaxis of hFL161/hTERT. Migration assay was assessed by a standard Transwell® migration system using Chemicon® ECM 508 assay kit (Millipore® AB, Solna, Sweden). Migration of cells towards the recombinant human chemokine ligands CXCL9 (0.1, 0.5 and 1.0 µg/ml), CXCL10 (0.02, 0.05 and 0.1 µg/ml) and CXCL11 (5, 10, and 10 ng/ml), and combined cocktails of the above (lower and high concentration of each ligand) were studied.

Characterization of cells (Paper I – IV)

Cultured cells were tested for hepatic, cancer, hematopoietic, viral and mesenchymal cell markers in early and late passages using four different assays: (a) immunocytochemical analysis, (b) qPCR, (c) flow cytometry, (d) biochemical staining, (e) electron microscopy and (f) live liver cell assays – cytokines, gene profile, *in vitro* functionality and matrix metalloproteinases (MMPs).

Immunocytochemical staining (Paper I-IV)

Immunocytochemistry (ICC) is a common laboratory technique that uses antibodies that target specific peptides or protein antigens in the cell *via* specific epitopes. These bound antibodies can then be detected using several different methods. ICC allows researchers to evaluate whether or not cells in a particular sample, express the antigen in question. In cases where an

immunopositive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen or whether passaged cell population maintain native antigens.

Cells were cultured on chamber slides (Becton Dickinson, USA). The medium was changed on a regular basis until the cells became 90% confluent. Immunoperoxidase staining was carried out using ImmPRESS™ REAGENT KIT anti-mouse Ig (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

qPCR for cells in culture (Paper I, II, IV)

Before performing *in vivo* experiments, one needs to be sure about the functional status of cells at the gene level. Hence, we obtained this information related to human hepatocyte specific transcription factors using PCR technology on cultured cells.

Total RNA was isolated from the respective cultured cells from the study using DNA/RNA/Protein isolation kits. RNA was extracted according to the manufacturer's instructions. The RNA concentration was determined by UV absorbance at 260 nm using a DU 730 spectrophotometer (Beckman Coulter, CA, USA).

PCR products were analyzed by gel electrophoresis in 2% agarose gels, stained with ethidium bromide, visualized on a the Gel Doc XR documentation system (Bio Rad; Sundbyberg, Sweden) and documented using Quantity One 1-D analysis software.

Flow Cytometry (Paper I-IV)

This procedure is chosen to characterize and quantify a specific cell population may be from mixed cell populations. It allows use of large sample sizes to obtain maximum accuracy in less time with less effort. Cells were analyzed on a FACS (Becton Dickinson, USA). Fluorescent signals from 10,000 cells were counted and the percentages of positively stained cells were recorded. Data acquisition and analysis were performed using CellQuest (Becton Dickinson).

Biochemical staining (Paper I, IV)

Glucose 6-phosphatase (G-6-Pase) is an enzyme that hydrolyzes glucose-6-phosphate resulting in the creation of a phosphate group and free glucose. This catalysis completes the final step in gluconeogenesis and glycogenolysis, which are important functions of hepatocytes in homeostatic regulation of blood glucose levels. “Oil-red O” is a fat-soluble dye used for staining neutral triglycerides and lipids on sections. The cells were counterstained with hematoxylin and mounted in aqueous mounting media (ImmunKemi, Stockholm, Sweden). Stained slides were examined under an Olympus microscope (Olympus, Hamburg, Germany). Both these stainings are used for detecting the functional status of the hepatocyte (Paper I) and CD271 cell populations (Paper IV). Hepatocyte functional markers G-6-Pase and glycogen were confirmed in transfected cells as described earlier (Rutenburg, Kim et al. 1969, Ott, Rajvanshi et al. 1999).

Electron microscopy (Paper IV)

Morphological characterization of the cells isolated for Paper IV (human liver stellate cells) was performed using electron microscopy. Isolated cells from adult and fetal livers in suspension (cells released by collagenase treatment) were fixed overnight in a mixture of 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.02% sodium azide, in cacodylate buffer, pH 7.2. Post-fixation with 1% osmium tetroxide and 1% potassium hexacyanoferrate was followed by *en bloc* contrasting with 0.5% uranyl acetate in water. Dehydration and embedding in epoxy resin of pelleted cells were performed according to routine procedure. Ultrathin sections were contrasted with uranyl and laid before examination in a LEO 912 AB Omega transmission electron microscope. Digital images were recorded with an Olympus-SiS Veleta CCD camera.

Live liver cell assays

Functionality of cultured cells was analyzed using different techniques as follows:

***Cytokine profile determination of cells* (Paper III-IV)**

The supernatants of cultured cells were collected. Quantification of inflammatory cytokines and chemokines was performed using Multi-Analyte ELISArray Kit (SA Biosciences Corporation, Frederick, MD, USA). Human cytokine levels in supernatants from cell cultures were measured with a sandwich enzyme linked immunosorbent based assay (ELISA) according to the manufacturer's protocol.

Genetic profiling of cells **(Paper IV)**

The genetic profile of cultured cells was determined by qPCR analysis. Cells for RNA extraction were collected and stored in RNAprotect cell reagent (Qiagen) and extracted using Qiagen's RNeasy Plus Mini Kit according to protocol. 1 µg of total RNA was converted to cDNA with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the protocol. Assays for the different genes were acquired from Life Technologies / Applied Biosystems. The qPCR was carried using a Gene Expression Master mix (Life Technologies /Applied Biosystems) in 10 µl reactions with 25 ng/well cDNA. The following cycling conditions on the 7900HT Sequence detection system (Applied Biosystems) with SDS 2.4 was used, 50 degrees for 2 min; 95 degrees for 10 min; 40 amplification cycles each consisting of 95 degrees for 15 seconds and 60 degrees for 1 min. The relative amounts of genes were compared with the reference gene: β-actin. This work was performed at the Sahlgrenska Academy genomics core facility.

In vitro functionality testing of cells **(Paper I-IV)**

Usually cultured hepatocytes are known to lose functional property over a few passages so it is of utmost importance to check the functionality status before transplantation. Cell lysates were prepared using a cell extraction buffer (Cat. No. FNN0011; Invitrogen, Stockholm, Sweden). Protein and urea concentrations were measured using the Bradford™ method and a quantitative colorimetric urea assay (Levine, Leon et al. 1961), respectively. The ammonia content and SOD activity was measured using a quantitative colorimetric assay kit (AA0100, Sigma) and a spectrophotometer-based assay kit (OxisResearch™, Foster city, CA, USA), respectively.

Metalloproteinases produced by cells **(Paper III-IV)**

A metalloproteinase, or metalloprotease, is any protease enzyme whose catalytic mechanism involves a metal. The hFLCs were tested for production of various MMPs. Cell supernatants from different passages were centrifuged at 3000 rpm for 15 minutes and stored at -80°C until further use. Human MMP-Array (RayBiotech, Inc.) was used to measure MMPs. This ELISA was quantified for gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, -8, and -13), stromelysins (MMP-3 and -10), and endogenous inhibitors [e.g. tissue inhibitor of metalloproteinase (TIMP-1, -2 and -4)]. The array membranes were processed according to manufacturer's protocol. The culture medium was used as negative control and each sample was assayed 3 times and the mean value of the measurements was used. The intensities of chemiluminescence signals of different MMPs were quantified by densitometry. The intensities of the positive control were used to normalize the results for the different membranes being compared. The chemiluminescence signal was detected using a Bio-Rad digital imaging, and densitometry data performed by ImageQuant 5.1.

Animal studies (Paper I- IV)

To prove the efficiency and pattern of distribution engraftment and the cell survival with sustained functional characteristics *in vivo*, one needs to perform animal experiments. Here we have used immunodeficient nude mice for the transplantation experiments since they represent the ideal recipient since they can accept xeno-cells to a certain extent of time without rejection. Also, it is easy to handle nude mouse model compared to other mouse models.

The animal experiments were approved by the animal ethics committee at experimental biomedicine (EBM), Gothenburg University and were performed in accordance with national and institutional regulations. BALB/c or C57BL/6 nude mice were purchased from Taconic, Denmark and Jackson USA. Animals were maintained in a 12-hour light/dark cycle maintained by automatic timers.

Transplantation of human liver cells into nude mice (Paper I-IV)

We developed and used a liver injury model using nude mice to study the engraftment of transplanted cells. Liver injury was induced in 6-8 weeks old mice either by intraperitoneal injection of retrorsine (70 mg/kg) (Paper I) or D-galactosamine (D-gal) (0.7 g/kg, Sigma, Stockholm, Sweden) (Paper II-IV) injection (Nowak, Ericzon et al. 2005). Three weeks after retrorsine (cytotoxic pyrrolizidine alkaloid inhibits endogenous hepatocyte proliferation) injection or 36 hours after D-gal (hexosamine derived from galactose, which is hepatotoxic causes fulminant hepatic liver) injection, all animals were subjected for partial hepatectomy (30-40%). This was followed by trans-splenic hepatocyte transplantation over 10-15 seconds in 200 μl of DMEM. In the chemokine study, the liver parenchyma was subjected to multiple injections of chemokines before LCT. The total operative procedure was performed under isoflurane anesthesia. After securing homeostasis, the abdominal incision was closed and the animals were monitored closely until recovery. In addition, sham operated nude mice were injected with 200 μl of DMEM medium alone.

Post transplantation, organ retrieval and analysis (Paper I-IV)

Mice were sacrificed 4 weeks after transplantation. The liver was excised from each animal. Biopsies of 1-2 cm^3 from each liver were snap frozen in liquid nitrogen and stored in -80°C until used for qPCR and immunofluorescence analysis. The rest of the liver tissue was fixed at 4°C for 24 hours in 4% buffered (pH 7.4) formalin to be stained afterwards for further protocols of stainings.

Immunohistochemical staining **(Paper I-IV)**

Immunohistochemistry (IHC) refers to the process of detecting antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.

Fresh frozen sections (4-5 μm thickness) of adult and fetal liver were used. IHC was performed using the biotin-peroxidase complex method. The slides were then processed as per standard protocol (Joshi, P et al. 2012). Experiments were accompanied by negative and positive control staining to detect possible nonspecific signals. Secondary controls were processed by replacing the primary antibody with antibody diluents only to detect possible nonspecific signals in the staining. Liver tissues from healthy and sham transplanted mice were used as negative controls, whereas human liver was used as positive control.

Immunofluorescence staining **(Paper I-IV)**

Immunofluorescence is a common technique to detect the distribution of single or multiple proteins simultaneously. In this technique, the antigen specificity of fluorescently conjugated antibodies is used to target fluorescent dyes to specific biomolecular target within a cell. Thus, allowing visualization of the distributed target molecule in the sample. Final results may vary depending on the fixation method, primary or secondary antibody concentrations and on many other parameters.

Five μm thick cryosections were air dried and fixed in ice cold 30% acetone in methanol for 10 min, and was further analyzed by immunofluorescence as per our earlier study (Joshi, P et al. 2012) using antibodies specific for human hepatocytes. Sections were counterstained with DAPI as before, mounted with aqueous mounting medium (Vector Laboratories), and examined under the fluorescence microscope (Olympus). As a positive control, sections from human cancerous liver tissue or normal human liver tissue were used. Furthermore, this technique was used to perform cell quantification assays.

Fluorescence in situ hybridization (FISH) **(Paper I)**

FISH is a cytogenetic technique is used to detect and localize the presence of specific DNA sequences in chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosome. FISH is often used for finding specific features in DNA for use in gene therapy, medicine, or for species

identification. Thus, it can be used to detect transplanted human cells within a mouse liver, which we took advantage of, using a human-specific DNA probe. Slides were viewed with an Olympus, ultraviolet lamp-equipped microscope carrying a triple band pass filter unit (Chroma Technology, Brattleboro, VT).

Enzyme Linked Immunosorbent Assay (ELISA) **(Paper I-IV)**

ELISA is a test that uses antibodies and color change to identify an antigen or targeted substance. It is a popular wet-lab analytic method of biochemistry assay that uses a solid-phase enzyme immunoassay to detect the relative or absolute quantity for the substance of interest e.g., an antigen, in a liquid sample or wet sample.

It is a very sensitive method and can detect low levels of protein in a given sample. In this thesis, it was used to quantify human ALB in transplanted mice sera. Sera taken from transplanted animals at four weeks were measured for human ALB (Bethyl Laboratories, Inc., Montgomery, TX) according to manufacturer's protocol. Human ALB concentration in serum samples of Tx, sham and normal animal groups was measured (absorbance) at 450 nm using an ELISA plate reader (Synergy H4, Hybrid Reader, Biotek).

MSCs are known to produce high levels of various cytokines and chemokines. Relative quantification of inflammatory cytokines and chemokines in cell culture supernatants was performed using Multi-Analyte ELISArray Kit (SA Biosciences Corporation, Frederick, MD, USA). In addition, human c-Met concentration in liver lysates was similarly measured using a sandwich ELISA kit (Invitrogen, Sweden).

***Western blot (WB) analysis* (Paper I-IV)**

WB is a widely accepted analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. Tissue lysates of all transplanted animals were used to detect the presence of transcription factors by standard WB. The WB bands were quantified by densitometry using the Quantity one (4.6.9) Image Analysis software (Bio-Rad, USA). Tissue samples of 10-15 mg from all transplanted animals were used to prepare lysates in RIPA lysis buffer. The protein concentration of each sample was determined by the Bradford™ method (Bio-Rad, Hercules, CA). A standard protein concentration of 30µg/ml was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred onto PVDF membranes (25 mA for 2 hours at 4°C; Bio-Rad, Hercules, CA). Proteins were detected by chemiluminescence (Amersham Biosciences AB, Uppsala, Sweden) based technique with the membrane finally exposed to an X-ray film (Amersham Biosciences AB, Uppsala, Sweden). The WB bands were quantified by densitometry using Quantity one (4.6.9) Image Analysis software (Bio-Rad, USA) and standardized to respective β-actin band to correct possible loading errors.

***qPCR TaqMan*® (Paper I-IV)**

Quantitative real time PCR (qPCR) is one of many variants of polymerase chain reaction. It is used to qualitatively detect gene expression through the creation of complementary DNA (cDNA) transcripts from mRNA.

Total mRNA was isolated from liver tissue from transplanted mice and human liver, according to the manufacturer's protocol (Norgen Biotek Corp, Canada). Samples obtained from mice were run in triplicate for qPCR. The cDNA samples were subjected to qPCR amplification using primers specific to human ALB, AFP, CK-19, CYP3A4, CYP3A7, HNF-1α, HNF-1β, HNF-4α and G6PD. The G6PD gene was used as the reference gene.

Quantification of engrafted hFLCs in mouse liver **(Paper I-IV)**

Engrafted and proliferating human liver cells in mouse liver were counted in serial sections of 5 μm thickness. Fresh frozen sections were subjected to single staining of human ALB and double staining of human ALB/Nuclei or Ki67/CK8 antibody. We counted cells from three to five portal or central regions on each section of the liver of each mouse in the different groups. At a 40x objective magnification, the number of engrafted cells in a 1 mm^3 of tissue was extrapolated from the observed sections.

Statistical analysis (Paper I-IV)

Data is presented as mean values with standard deviations. Statistical analysis of *in vitro* functionality and human ALB in serum was performed using the two-tailed Student's *t* test. Densitometry and quantification of transplanted cells were analyzed using One-way ANOVA followed by Tukey's test for post correction. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc, Chicago, USA) and GraphPad Prism version 4.0 respectively.

Ethical permits (Paper I-IV)

All experiments were approved by the University Ethical Board and presented to the evaluation committee before start of studies for the thesis.

RESULTS

Results

Paper I (Co-transplantation [CoTx] of hFLCs and hFLMSCs)

In vitro experiments

Characterization was performed using ICC and FACS. Human fetal hepatocytes showed all hepatocyte markers among which ALB expression was frequent and intense in hFHs, whereas human hFLMSCs expressed typical MSCs markers and were negative for several hepatocyte markers.

Fetal cells produced low amounts of functional parameters as compared to adult hepatocytes. In hFLMSCs supernatant, relatively high levels of IL-6 were found together with other pro-inflammatory cytokines (Paper I - Fig. 3).

In vivo experiments

Immediately after injection, we observed transient hypertension in all animals as a result of entrapped cells. We confirmed the presence of engraftment and repopulation with human cells in the experimental model by IHC and qPCR. Liver sections of the CoTx group showed colonies of hepatocytes synthesizing ALB, whereas hFLMSCs revealed only a few cells positive for human ALB. The livers of the CoTx group showed high numbers of cell colonies expressing human-specific antigen near the portal region.

In the CoTx group, intense human-specific mRNAs expression was detected compared to the fetal hepatocyte transplantation (FHTx) group and the group receiving only hFLMSCs (MTx). Human ALB concentration in the CoTx group

was the highest compared to the FHTx ($p<0.01$), whereas it was not detected at all in the MTx group. A similar pattern was seen in serum c-Met concentration levels.

The magnitude of liver repopulation by transplanted human cells carrying the hepatocyte markers was the highest in the CoTx group. The number of double positive cells (H. nuclei^{+}/ALB^{+}) was significantly higher in the CoTx group compared to ($p<0.001$) the FHTx and the MTx group. In the MTx group more single positive (H. nuclei^{+}/ALB^{-}) cells were found. The Ki67 and CK-8 double positive cells were fewer in all groups, suggesting that transplanted cells have a limited ability to proliferate (Paper I-Table III).

Paper II (SV40-LT transduced hFLCs)

***In vitro* experiments**

Phenotypic characterization of SV40 large T antigen-immortalized hFLCs (SV40LT-hFLCs) showed expression of hepatic and biliary markers in almost all cells, both in early and late passages. Cytochrome P450 (CYP3A4/7) and transcription factors were also expressed in these cells.

As determined by immunocytochemistry, the SV40 large T antigen expression in SV40 LT-HFL cells was consistent during cell culture, suggesting a stable integration into the host genome of the plasmid encoding the SV40 LT gene. In addition, the transfected cells did not stain positive for cancer-associated markers. Furthermore, we found that the transfected cells expressed the liver specific marker G-6-Pase and they could store glycogen.

Hepatic progenitor cell markers were detected in early passages of SV40LT-HFL cells (p11, p22), using flow cytometric analysis. However, the cells did not express the MSCs and hematopoietic stem cell markers. However, immortalized hFLCs were positive for some of these markers, including ALB, HNF-4α and CYP3A4/7.

***In vivo* experiments**

SV40-LT transfected hFLCs differentiated into mature hepatocytes *in vivo* and could be visualized using IHC. Repopulation of the bile ducts by human progenitor cells was observed along with several clusters of cells expressing human CK-8, human c-Met and cells expressing the hepatocyte-specific antigen in all transplanted mice. Furthermore, the transplanted cells did not express the tumor suppressor marker p53 or the cancer cell marker Ber-EP4. Varied levels of human ALB were measured (03.39-221.30 ng/ml) compared to the negative controls (0±0 ng/ml), and lower amounts of CYP3A4 were detected in the transplanted animals.

Paper III (hTERT transduced hFLCs and chemokines)

***In vitro* experiments**

The characterization of hFL161/hTERT using ICC demonstrated the necessary hepatocyte markers (Paper III - Fig. S1). However, the level of urea and ammonia were significantly lower than in adult hepatocytes cultures.

The chemokine receptor mapping showed that the two receptors that were commonly expressed in both fetal and adult livers were CXCR3 and CXCR4. We observed increased migratory behavior of both cell types towards the corresponding ligands to CXCR3 (CxCL -9, -10 and -11) on human hepatocytes.

***In vivo* experiments**

Repopulation of injured livers transplanted with hFL161/hTERT cells was observed in mice receiving cocktails of chemokine ligands (CXCL -9, -10 and -11). Furthermore, human cells were shown to be positive for CK-18 and -19 near the bile ducts. The number of double positive cells expressing CK-8/Ki67 was not significantly different among any transplanted groups (IV-VII). Cell colonies expressing human nuclei and ALB were significantly higher in Group VI. Furthermore, in Group V, we observed integration of hFL161/hTERT in mouse bile ducts (Paper III - Fig. 3).

Human ALB concentration in serum was the highest in Group VI (74.8 ± 0.9), followed by Group V (70.9 ±0.70). The presence of human-specific protein and gene expression in injured mouse livers were detected in mouse liver using WB and qPCR. WB data showed that animals receiving the cocktail C-2 (Group VI) and the C-1 (Group V) had a 3.05 fold and a 1.65 fold increased protein expression of CYP3A1 compared to the chemokine control (Group VII), respectively.

The data obtained from the injected cells (hFL161/hTERT), the livers of transplanted animals and the human liver, showed that the transplanted

animal livers had a pattern similar to the expression of the injected cells, not to that of the cells in human liver. Cytochrome P450 (CYP3A4 and 3A7) expression was not detected in the transplanted groups.

At 4 weeks after cell transplantation, numerous large clusters of double positive hepatocytes (H. nuclei^{+}/ALB^{+}) were present in the transplanted groups. In addition, we also found single positive cells (H. nuclei^{+}) without ALB. The location of the engrafted cells in Groups V and VI were the same, with scattered engrafted cells in the centrilobular and periportal region. Group VI had the highest magnitude of liver repopulation overall.

However, transplanted cells behaving as hepatocytes produced significantly higher ALB in Group VI *vs* VII (p=0.0003) and also in Group V *vs* IV (p<0.05). There was no significant difference observed in ALB negative cells or mitotic human cells among transplanted groups (Paper III - Fig. 6).

Paper IV (human liver stellate cell)

***In vitro* experiments**

IHC staining for CD271 antigen of normal human adult liver demonstrated a discontinuous pattern with perisinusoidal stellate shaped cells, whereas in fetal livers, the cells were observed uniformly throughout the liver.

On average, isolation of CD271^{+} cells by both MACS and FACS methods yielded similar numbers of cells. Based on trypan blue staining, the viability of isolated

$CD271^+$ cells from both liver sources was approximately 96±4% after immunomagnetic isolation.

In culture, the fetal $CD271^+$ cells developed star-shaped morphology and had some lipid-containing droplets. After about 6 passages, cells in culture developed a spindle-shape and started to resemble myofibroblasts and had fewer lipid-droplets. On the other hand, only freshly isolated $CD271^+$ cells from adult livers had star-shaped morphology and abundant lipid droplets, but became spindle-shaped after >3 cell culture passages. Cultured cells under TEM showed evidence of co-location between lipid droplets and glycogen deposits with the droplets being surrounded by glycogen (Paper IV - Fig. S1).

$CD271^+$ cells isolated from human livers expressed both stellate and MSCs markers. FACs analysis demonstrated that although freshly isolated fetal $CD271^+$ cells did not express stellate cell markers, adult $CD271^+$ expressed all the markers of stellate cells and were positive for GFAP (strong expression), desmin (strong expression), vimentin, and alpha-smooth muscle after one week in culture. Although, they weakly expressed the stem cell markers, very strong expression for the CD90 was found. However, the cells did not express any hepatic markers and the phenotype of $CD271^+$ cells was similar to that described for MSCs. ON the other hand, the $CD271^-$ cells were found to be positive for the hepatic markers.

Analysis of known stellate cell markers by qPCR showed that fetal liver derived-$CD271^+$ cells strongly expressed mRNA for CD271, vimentin, desmin, alpha smooth muscle and GFAP as compared to $CD271^-$ cells.

Metalloproteinases produced by CD271$^+$ cells were evaluated using immunoblot and showed the highest expression of TIMP-2, followed by MMP-3, TIMP-1, and MMP-1 respectively in CD271$^+$ cell supernatants. There was a weak detection of MMP-10, and MMP-13, but the cytokine profile for CD271$^+$ cells gave similar results from adult and fetal liver. The production of IL-8 was the highest as compared to other measured cytokines.

***In vivo* experiments**

Transplanted human fetal CD271$^+$, were detected mainly in the hepatic parenchyma within the perisinusoidal space. CD271$^+$ cells were also found located within the stroma of portal tracts found adjacent to the wall of the portal vein (Paper IV – Fig. 4). Immunofluorescence double staining for anti-human nuclei and GFAP, demonstrated double positive adult liver CD271$^+$ cells in the perisinusoidal area, confirming the presence of human HSCs. In addition, anti-human nuclei and SMA double positive cells were found in portal tract vessels and in cells lining the hepatic vein and artery. Transplanted CD271$^-$ cells were positive for the hepatocytes markers CK-8, CK-18 and for the hepatocyte-specific antigen.

GENERAL DISCUSSION

General discussion

We have used a modified model of liver injury by performing a 30-40% PH in order to induce enough cytokine storm for regenerative response. At the same time it has preserved a substantial (total) volume of the native liver mass which facilitated low intraoperative mortality of models and mimicked closely to the clinical situation.

Do MSCs have a facilitator effect during hepatocyte transplantation?

We co-transplanted (CoTx) highly proliferative hFHs together with "facilitator cells" (hFLMSCs), and our results demonstrated that mesenchymal stem cells (MSCs) improved the engraftment of hepatic progenitor cells. The CoTx group showed a higher capacity for hepatocyte engraftment (the total cells engrafted per mm^3 was estimated to be 81,000±2003) in comparison with fetal hepatocytes were transplanted alone (the hFHTx group: 58300±8800), or the MSCs transplantation alone (the MTx group: 1329±156) (Paper I - Table III).

In clinical hepatocyte cell transplantation engraftment of hepatocytes in the diseased liver is influenced by many factors such as deficiency of nutrients, hypoxia, oxidative stress, inflammatory response and fibrosis. The environment is harsh in the damaged liver, where vascular structures are impaired and trophic support is lacking. Death of transplanted hepatocytes further intensifies the harshness of the microenvironment by priming the immune and the inflammatory response. It is therefore important to optimize LCT conditions so

that transplanted cells can withstand these severe conditions, especially during the acute phase after cell infusion. Thus, successful LCT will depend on the use of selective and highly proliferative cells (i.e. use of progenitor/stem cells or transduced cell lines), use of preconditioning regimens (i.e. chemokines priming) and *in vivo* bioengineering (i.e. co-transplantation with facilitator cells). Furthermore, transplanted cells must also have a selective engraftment advantage when introduced into the ischemic environment that provides them with a time-window long enough for the transplanted cells to acclimatize and engraft. For these reasons, we attempted a novel approach using a heterogeneous cell population for transplantation; and were able to demonstrate an improved engraftment.

Our immunocytochemical analysis showed expression of several liver specific markers in hFHs, however, hFLMSCs did not show expression of ALB, CK-19, and hepatocyte specific antigen, although they did express c-Met, CK-8 and CK-18. *In vitro* measurement of hFHs and hFLMSCs cell cultures for ammonia, urea content, and SOD activity demonstrated that, in general, these cells had significantly lower values as compared to adult hepatocytes, indicating the immature nature of these fetal cells. This is not surprising, however, since energy requirements in fetal life are fulfilled *via* maternal blood. At birth this supply is interrupted and replaced by external supply, and thus the detoxification system evolves after birth (Barr, Bishop et al. 2007).

We observed that only a low number of MSCs expressed hepatic markers, which indicates that MSCs may not have the capacity to trans-differentiate into

hepatocytes, as has been suggested by other studies (Gonelle-Gispert 2007). For these reasons, we would like to argue that if hFLMSCs under the appropriate conditions still do not differentiate into hepatocytes, then the probability of trans-differentiation of MSCs from other tissues into hepatocytes might even be lower.

Despite the low hepatic expression, transplanted MSCs were beneficial. An important question that needs to be addressed is: by what mechanism did these cells augment hepatocyte engraftment? Our preliminary data using ELISA demonstrated that supernatants at various passages during hFLMSCs culturing showed significantly higher levels of IL-6 and IL-8, but low levels of TNF-α and IL-1. It has been reported that MSCs secrete high levels of IL-6 as an anti-inflammatory cytokine, which is mediated through inhibitory effects on TNF-α and IL-1 (Rijneveld, Florquin et al. 2001, Heinrich, Behrmann et al. 2003). Thus, higher levels of IL-6 and IL-8 produced by MSCs may counterbalance the hepatocyte transplantation-induced liver inflammation caused by pro-inflammatory cytokines produced by Kupffer cells and infiltrating neutrophils (Krohn, Kapoor et al. 2009). Furthermore, metalloproteinases produced by MSCs may induce vascular permeability allowing larger numbers of hepatocytes to translocate quickly through the sinusoids and integrate efficiently into the liver parenchyma (Eum, Lee et al. 2006, Bauer, Burgers et al. 2010). It is important to state that in our experience, MSCs derived from bone marrow did not significantly improve engraftment as compared to hFLMSCs (unpublished data). This finding reflects

that tissue-specific factors produced by MSCs may play an important role in efficient engraftment.

It is probable that for future successful clinical hepatocyte transplantation, liver-derived MSCs may be the best candidate for improving engraftment of the infused hepatocytes. We are currently elucidating the mechanisms by which hFLMSCs augment hepatocyte engraftment.

Can lentivirus (SV40-LT) transduced hFLCs maintain phenotypic and *in vivo* functionality over several passages?

Our results demonstrated that transfected cells expressed the introduced genes and their subsequent proteins *in vitro*. However, after transplantation, we found that liver tissue of (cell transplanted) mice expressed low amounts of CYP3A4, but showed no expression of CYP3A7, indicating that the *in vivo* conditions may have been conducive for differentiation of the immature hFLCs.

The oncogenic potential of the SV40 large T antigen resides in part in its ability to bind and inactivate many of the activities of the tumor suppressor p53 (May and May 1999, Ali and DeCaprio 2001, Pipas and Levine 2001). However, our SV40 LT Ag transfected cells did not stain positive for p53. Furthermore, transplanted cells *in vivo* did not demonstrate tumor formation at 4 weeks after transplantation, or expression of p53 or Ber-EP4 (unpublished data). It is important to state that further long term *in vivo* experimental studies are required to evaluate the tumorigenic potential of the present cell line.

Thus, we have succeeded in establishing *in vitro* expandable hFLCs by means of immortalization and without inducing an altered phenotype and disrupting their differentiation potential. This cell line would facilitate studies on cell engraftment and differentiation within the hepatic parenchyma. In most cases, murine hepatic stem cell lines have been used to study immortalization and transduction *in vitro* (Kim, Sung et al. 2000, Zhang, Guckian et al. 2002). However, the characteristics of murine stem cells cannot be extrapolated to their human counterparts, therefore it is important to establish human hepatic progenitor cell lines to study the molecular events involved in their proliferation and differentiation *in vitro,* as well as their fate *in vivo* after transplantation.

Furthermore, we studied the expression of two important transcription factors: HNF-4α and HNF-1α. HNF-4α is required for the PXR and CAR-mediated transcriptional activation of CYP3A4 and is a transcription factor that is involved in the regulation of the expression of several liver specific genes (Akiyama and Gonzalez 2003). HNF-1α (hepatocyte nuclear factor 1, homeobox B), is encoded by the transcription factor 2 gene, a liver-specific factor (Akiyama and Gonzalez 2003). We found that the SV40-transduced cell line expressed *both* these important transcription factors. Therefore, these immortalized cells may be useful for the development of diagnostic tools for toxicity studies.

Can temporary priming of the liver parenchyma with selective chemokine ligands enhance the engraftment of transplanted fetal hepatocytes?

We demonstrated *in vitro* that immortalized fetal hepatocytes with hTERT, efficiently migrated towards different concentrations of the CXCR3 ligands CXCL -9, -10 and -11. Cocktails of these chemokines induced increased cell migration, which may imply a synergistic effect of the three chemokines. Furthermore, using a nude mouse model with D-galactosamine-induced liver damage showed an abundant number of ALB-positive transplanted cells in the mouse liver of chemokine cocktail-treated groups, as compared to sham and control groups.

Our results also demonstrated that engraftment of hepatocytes was enhanced with the chemokine priming. It is likely that increased adhesion of the transplanted cells to the chemokine ligands may have resulted in better survival and retainment of these cells in the liver, which may in turn have led to the robust repopulation. Results from WB and qPCR analysis demonstrated that the transplanted cells expressed several important hepatic transport proteins and hepatic transcription factors (Nagy, Bisgaard et al. 1994, Greenbaum, Cressman et al. 1995). High engraftment of human hepatocytes in knockout mouse models can be achieved by serial transplantation, but it involves major surgical issues (Tateno, Yoshizane et al. 2004, Turrini, Sasso et al. 2006, Azuma, Paulk et al. 2007, Katoh, Sawada et al. 2007). The advantage of our procedure is that, a single injection of 2 x 10^6 hFL161/hTERT is

sufficient to achieve a robust repopulation, taking advantage of the important role of chemokines in cell-homing, retention, and engraftment of cells. Based on the results obtained, we believe that these chemokines may have favored retention and survival of transplanted hepatocytes expressing the corresponding chemokine receptors. In fact, CXC chemokines have been shown to induce proliferation of hepatocytes both *in vitro* and *in vivo* (Colletti, Green et al. 1998, Clarke, Kuboki et al. 2009). Furthermore, treatment of mice with CXC chemokines increased hepatocyte proliferation and liver regeneration after partial hepatectomy (Ren, Carpenter et al. 2003). The successful clinical outcome of hepatocyte cell transplantation depends on the sufficient repopulation of the liver. Wider use of clinical hepatocyte transplantation can be a reality if strategies improve repopulation are established which encourages cell survival during and after cell transplantation. Supplying chemokines in combination with the hepatocytes may therefore be one strategy to increase repopulation.

Interestingly, results obtained from the various transplanted groups demonstrate that liver injury and partial hepatectomy (Chan, Cheng et al. 2012) or liver injury and exogenous chemokine injections alone may not be sufficient to induce augmented engraftment of transplanted cells. It is reported that levels of CXC chemokines are increased 3- to 5-fold after 70% hepatectomy, indicating that PH results in only low to moderate levels (Colletti, Green et al. 1998). In the present study, animals showing the highest engraftment cell levels were those with liver injury + PH + additional doses of exogenous hepatic chemokines: CxCL -9, -10 and -11.

Our results also demonstrate that chemokines are essential components not only in the process of engraftment, but also in proliferation of hepatocytes, as evidenced by the presence of several $Ki67^+$ human cells even in the hostile environment of chemically damaged livers.

Generally, chemokines attract inflammatory cells (Qin, Rottman et al. 1998). Partial hepatectomy which stimulates release of chemokines, results in infiltration of inflammatory cells in the liver (Clarke, Kuboki et al. 2009). Therefore, one would expect increased damage or destruction of livers after additional injection of exogenous chemokines. Although we did not encounter this problem in the present study due to use of nude mice, we believe that the small volumes of injected chemokines quickly dispersed into the parenchyma and thereby preventing the formation of a gradient that lead to uncontrolled and prolonged action of the chemokines, which may be potentially harmful (Clarke, Kuboki et al. 2009). Furthermore, since the chemokines quickly disperse into the liver lobes, it is important that the cells are transplanted immediately after injection of chemokines to maximize the effects of these proteins.

Several animal hepatocyte transplantation models have shown low survival rates of engrafted cells. In rats, transplantation of $2x10^7$ hepatocytes resulted

in engraftment of approximately 0.5% of the total recipient liver cells (Allen and Soriano 2001) and the hepatocyte loss occurred during the early stages of transplantation (Gupta, Gorla et al. 1999, Joseph, Malhi et al. 2002). Usually, the major loss of transplanted cells is thought to be due to the failure in entering sinusoids and crossing sinusoidal endothelial barriers (Gupta, Gorla et al. 1999). In rodent models, less than 30% of the transplanted cells are found within the liver parenchyma after 24 hours (Gupta, Gorla et al. 1999) whereas in our study, a robust engraftment was recorded (66% and 75% engraftment in Groups V and VI, respectively) after 4 weeks of transplantation in mice livers.

Transplantation of stem and progenitor cell populations, rather than mature hepatocytes, has been recently investigated, exploiting their known proliferative potential. Stem/progenitor cells are minimally immunogenic and readily cryopreserved, but are small and engraft with lower efficiency than larger mature cells (Susick, Moss et al. 2001, Weber, Groyer-Picard et al. 2009, Khan, Shaik et al. 2010, Turner, Mendel et al. 2012). On the other hand, clinical trials with transplantation of hFLCs have revealed no evidence of emboli formation and no need for immunosuppression. It has also shown improved end-stage liver disease scores, longer survival of seriously ill patients, and improved liver functions in all transplanted patients (Khan, Shaik et al. 2010).

Although we tested the effects of CxCL -9, -10 and -11 in the present study, chemokine-ligands to other receptors expressed on hepatocytes such as SDF-1 can also be tested. We believe that this approach to establish “humanized livers” in mouse models will be useful for pharmaceutical and virological

studies. In addition to lab models, it might represent a potential regimen to practice in the clinical set up with a promising prognosis of transplanted hepatocyte survival and functional proliferation. The efficiency and rapid action of these chemokines and lack of pro-inflammatory activity makes this approach an attractive strategy to augment repopulation of transplanted hepatocytes *in vivo*. Our newly established immortalized fetal hepatocyte population revealed morphological characteristics of primary hepatocytes in standard culture systems and expressed many liver enriched markers.

Can CD271 antigen in hFLCs be a marker for isolating the MSC precursors to HSCs, portal fibroblasts and hepatic vascular smooth muscle cells?

Our results demonstrate that $CD271^+$ cells are HSCs that secreted a series of molecules with pro-inflammatory (IL-6, IFN-γ, TNF-α), chemoattractant (IL-8, IL-1α and β), differentiation (GM-CSF), and immunoregulatory (IL-10) activities (Charles, Chou et al. 2013) as well as the T-cell growth factor IL-2, which indicate that $CD271^+$ cells in the liver may participate in inflammatory processes leading to fibrosis during liver injury.

HSCs are the major source of ECM in liver fibrosis (Friedman 1993). HSC activation is accompanied by a marked expansion in HSC number and expression of the tissue inhibitors of metalloproteinase (TIMP-1 and -2) (Iredale, Murphy et al. 1992, Knittel, Mehde et al. 1999). Activated HSCs are the major source of TIMPs during chronic liver injury and therefore, in addition to producing the excess matrix that characterizes fibrosis, HSCs might shift the

extracellular environment to one that favors matrix deposition (Iredale, Benyon et al. 1998, Knittel, Mehde et al. 1999). We found that cultured fetal and adult CD271$^+$ cells produced high levels of TIMP-1; TIMP-2 and MMP-1 and 3, indicating that these cells express the principal components of the fibrotic neomatrix, and may contribute to the hepatic myofibroblast pool.

HSCs also participate in modulating liver inflammation during liver fibrogenesis (Marra 1999) by producing a variety of pro-inflammatory cytokines (Friedman 1993).

It is reported that there are three major mesenchymal cells in the liver; HSCs, portal fibroblasts, and vascular smooth muscle cells [present in the walls of liver blood vessels] (Asahina, Tsai et al. 2009). HSCs, are found in the perisinusoidal space between sinusoids and hepatocytes in the adult liver (Takase, Leo et al. 1988). Portal fibroblasts are located in the connective tissue around portal vessels and bile ducts. In the current study, we found that fetal CD271$^+$ cells, when injected *in vivo*, were found in the perisinusoidal space and around portal vessels, but not in the walls of liver blood vessels in response to partial hepatectomy. This indicates that these cells were precursors of HSCs and portal fibroblasts, while adult CD271$^+$ were found lining the liver sinusoids *in vivo* and also found in the vessel walls of the portal vessels.

In the past, a seven-step method was used for isolation of HSCs (Knook and Sleyster 1976, Gressner and Schafer 1989, Maschmeyer, Flach et al. 2011). However, when the lipid content is low in HSCs, the density of these cells resembles liver sinusoidal endothelial cells, thus making the separation difficult

(Brouwer, Wisse et al. 1988). The technical difficulties limit the use of this procedure. In this study, we report an alternative that successfully uses CD271, as a marker for isolation of HSCs during immunomagnetic cell separation, generating reliable and reproducible isolations with high viability cell yields (96%). Until now, limited knowledge exists regarding HSCs except their vital role in liver fibrosis and cirrhosis (Tanaka, Minato et al. 1984, Gressner 1998, Pinzani and Gentilini 1999, Friedman 2000), however, the exact underlying mechanisms are not clear. Studies using isolated and cultured $CD271^+$ cells may be appropriate in further understanding of the mechanisms of HSCs in liver fibrosis. Moreover, general studies on cellular dynamics (e. g. activation, trans-differentiation, senescence) or its sustainable modulation (e. g. abrogation, reversal) in HSCs/MFB may be performed using $CD271^+$ cells. The role of CD271 *in vivo* during fibrogenic injury has not been determined. It is possible that CD271 activation may represent a potential mechanism to target HSCs for apoptosis and may provide a potential mechanism for the selective depletion of HSCs.

CONCLUSIONS

Conclusions

The major findings of this thesis are:

- hFLMSCs facilitates the engraftment of hepatic progenitor cells.
- SV40-LT transduced hFLCs provided a stable cell line with functional capacity.
- Preconditioning with chemokine ligands in appropriate doses can enhance hepatocyte cell transplantation results.
- The CD271 marker of hFLCs can be used for isolation of MSC precursors for HSCs, portal- fibroblasts and vascular smooth muscle cells. These cells provide a novel culture system to study human hepatic fibrogenesis, gene expression, and transcription factors controlling HSC regulation.

REFLECTIVE STATEMENTS

Reflective statements

This thesis addresses a few important factors that might be vital for clinical applications, but a lot of work remains to be done. To facilitate the development of cell-based therapies for the treatment of liver diseases, stem cell–derived hepatocytes will need to show more than a marginal capacity to engraft and proliferate in the liver after transplantation. Normal levels of engraftment, functions, expansion, relationship to other intrahepatic cells and structures will need to be proven. Also, transplantation studies shall correct liver disorders successfully in appropriate animal models. To be useful for transplantation, additional metabolic activities such as the ability to metabolize drugs, toxins, ammonia and conjugate bilirubin will be critical. It will not be enough to alter a relatively small percentage of the manipulated cells into “hepatocytes” because patients require transplantation of more than a billion cells at a time. The safety issues will not be proven enough by failure to identify tumors in a handful of immune-deficient rodent studies, and demands further detailed investigation with alternative techniques. In other words, a stable cell line developed in this thesis using SV40-LT antigen has shown to generate an ample amount of cells for transplantation, though the safety and efficiency of these cells in a clinical setting can’t be judged only from experiments using nude mice. As noted, such transplantation studies may take considerable effort since maturation after transplantation in the host liver may be required. Animal models that allow time for differentiation to occur after transplantation, such as models of metabolic disease, may be more appropriate to examine the functionality of stem cell–derived hepatocytes. Additionally, in such models, the

life of the animal is not in immediate jeopardy and the neo-hepatocytes will be allowed weeks, or even months to correct a liver defect, such as the hyperbilirubinemia [associated with the Gunn rat model of Crigler-Najjar syndrome type I], or any of the clotting factor deficiencies. So, precisely *what* characteristics do "derived hepatocytes" need to become effective surrogates for the real thing? It would be ideal to derive cells with characteristics identical to those of primary human hepatocytes in all aspects, but can we do with less? If we can generate cells that have some hepatic characteristics and are permissive *in vitro* for viral hepatitis infection, much could be learned about viral hepatitis biology and its treatment. Similarly, cells that could reproducibly metabolize drugs in a fashion similar to that of primary hepatocytes would revolutionize drug discovery and begin the process of "personalizing" health care (Fox and Strom 2008).

Currently, no consensus exists regarding a standardized method for measuring engraftment in the field of clinical hepatocyte cell therapy. The demonstration of engraftment and repopulation of the recipient liver by donor hepatocytes is still a major difficulty. In certain inborn errors of metabolism, the restoration of a metabolic defect after hepatocyte transplantation can be accessed from the serum concentration of a metabolite, but this may not provide reliable information about the number of surviving and functioning engrafted cells. Moreover, the distribution of the engrafted cells cannot be determined by this approach. Techniques such as short tandem repeat analysis: FISH or qPCR for the Y-chromosome sequences in cases of sex-mismatched hepatocyte transplantation can be performed on liver biopsies. Unless a new method is

established, it is not possible to draw reliable conclusions regarding engraftment levels or repopulation. It is therefore imperative that a universally acceptable standardized method for estimating donor hepatocyte engraftment is developed.

Fetal progenitor cells have shown great promise for safe proliferation *in vivo* and in liver regeneration. Bringing LCT from the laboratory bench to the bedside and test their therapeutic potential, will be a huge challenge for scientists and clinicians. This may be possible if a large number of functional hepatocytes from stem cells are created in a safe, reliable and reproducible manner, free from risks of xenobiosis. Trials in large animal models and careful dissection of the repair mechanisms will be critical. The best liver stem cell candidate shall need to be promoted and helped to overcome these obstacles, which stand between the lab bench and clinic. Due to *in vitro* manipulation of cells, causing unstable and unpredicted variations in transplanted cells, a special group of scientists tried to formulate recommendations. These recommendations include model selection for testing LCT and a character-checklist for transplanted cells (Sancho-Bru, Najimi et al. 2009), which is a promising start to begin a new hope in LCT.

Furthermore, regarding fetal hepatic stem cells, ethical barriers have been the limiting factor in many countries. Until these barriers have been overcome, the search for the most appropriate liver stem cell will continue and the promise to cure liver diseases with so-called the miraculous cell therapy will remain elusive (an unsolved puzzle).

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