GENETIC REGULATION OF HUMAN FETAL LIVER AND ITS CLINICAL APPLICATION

ANTONY SAGAYARAJ IRUDAYASWAMY

(M.Sc.)

NATIONAL UNIVERSITY OF SINGAPORE

2013

GENETIC REGULATION OF HUMAN FETAL LIVER AND ITS CLINICAL APPLICATION

ANTONY SAGAYARAJ IRUDAYASWAMY

(M.Sc.)

A THESIS SUBMITTED FOR THE DEGREE OF

MASTERS BY RESEARCH

DEPARTMENT OF MEDICINE

YONG LOO LIN SCHOOL OF MEDICINE

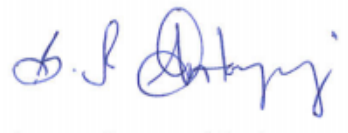
NATIONAL UNIVERSITY OF SINGAPORE

2013

DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information, which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



_____

Antony Sagayaraj Irudayaswamy

5th June 2015

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my mentor, **Associate Professor Dr. Dan Yock Young** for his continuous support during my Master's study and research; and for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me all the time – in my research and in the writing of this thesis. I could not have imagined having a better advisor and mentor for my Master's study. I am greatly indebted to **Professor Dr. Lim Seng Gee**, Department of Medicine, National University of Singapore for his expert advice and suggestions to my research during the candidature.

I must thank my lab mates, **Mrs Jaymie Lin**, **Mrs Nurhalisa, Dr. Zhouli, Mr. Peters Brain**, **Mr. Daniel Teo and Dr. Wang Yu** for all their help and valuable suggestions during lab meetings. I would also like to take this opportunity to thank my friends **Dr. Shriram Venkatesan** and **Late Mr. Dhayaparan Devaraj** for their support and friendship.

I wish to thank National University of Singapore for the Research Scholarship and National Medical Research Council financial aid to support this study.

Lastly, and most importantly, I thank my family (my parents, my wife Dr. Janani Manivannan and my Family memebers) for their unending love, care and support.

Dedicated to my father, who has recently passed away

due to kidney failure

TABLE OF CONTENTS

PUBLICATIONS

International Journals:

1. **A. Sagayaraj**, Zhou Lei, Hung Hau, KKH, PC Wong, Kenneth Kewk, Dan YY. "*Genetic regulation of fetal liver development"*. In preparation for submission to Development

2. NH Jumaat, **A Sagayaraj,** J Lin, SL Chan, J So, YY Dan. *"Novel human Gastric Progenitor Cells as potential Autologous Endodermal Cell Source for Liver Regeneration"*. In preparation for submission to Gastroenterology.

3. L Zhou, J Lin, **A Sagayaraj**, NH Jumaat, YY Dan. *"Immunopermissive Mouse Injury Model of liver cirrhosis and their potential for testing stem cell therapy".* In preparation for submission to Hepatology.

4. YY Dan, J Haque, **Antony Sagayaraj**, H Hau, N Teoh, Kim Riehle, Jaymie Lin, PC Wong, KKH JS Campbell, N Fausto. *"Long Term In-vivo Repopulation of Human Fetal Liver Progenitor Cell in Liver Injury".* (Manuscript under publication).

Conference poster presentations:

1. **Antony Sagayaraj,** *Dan Yock Young. *"Genetic regulation of progenitors cells in human liver development"* in "*Yong Loo Lin School of Medicine Graduate Congress"* held on 15th February 2012 at Centre for Life Science, National University of Singapore, Singapore.

2. **Antony Sagayaraj,** Zhou Li and *Dan Yock Young. *"Liver Progenitor Cell Niche in Human Fetal Liver and Symmetry in Cirrhosis"* in "*American Association for the Study of Liver Diseases (AASLD), The Liver Meeting"*, held on 4th-8th November 2011 at San Francisco, USA.

3. **Antony Sagayaraj,** Hung Hao and *Dan Yock Young.*"Genetic regulation of liver stem cells in human liver development"* in *"Asian Pacific Digestive Week (APDW 2010)"* held on 19th-22nd September 2010 at Kuala Lumpur, Malaysia.

4. **Antony Sagayaraj,** Hung Hao and *Dan Yock Young.*"Genetic regulation of progenitors cells in human liver development"* in *"International Anatomical Sciences and Cell Biology Conference (IASCBC 2010)"* held on 26th-29th May 2010 at University cultural Center, National University of Singapore, Singapore.

SUMMARY

Human liver progenitor cells are potentially useful for a wide variety of clinical applications such as transplantation, bioartifical liver, drug development and gene therapy. Current gaps in understanding of the key mechanisms that drive progenitor cells proliferation and maturation have limited the realization of their full potential. We aimed to determine the key message signals that drive progenitor cell proliferation and differentiation in developing fetal liver and determine their implication in regenerative medicine. Our aim is to determine the key message signals that drive progenitor cell proliferation and differentiation in developing human fetal liver and determine their functionality and application in regenerative medicine. Human fetal livers from 10 weeks to 24 weeks gestation were obtained with full consent. These samples were characterized with IHC, microarray, next generation sequencing and qRT PCR for growth factor, extracellular matrix and transcriptional factors. Comparison was made with adult normal livers. Using immunophenotyping and transcript signature, 5 phases were distinguished and categorized in fetal liver development. Fetal liver progenitor cell undergoes proliferation at 10 to 14 week gestation, from 14 to 18 weeks differentiation of fetal hepatic lineage - the hepatoblast form, in week 18 there are signs of hematopoietic lineage expression from liver and from week 20 onwards there is a sudden proliferation of EpCAM and Vimentin positive cells, which is seems to be a mesenchymal lineage.

EPCAM/CD44+ progenitor cells appear at 10 weeks and undergo surge in proliferation at 18-20 weeks of gestation before dwindling in frequency. The expression pattern of EPCAM corroborate that there are distinct phases such

as progenitor cell population surge at 10 weeks, hepatoblast proliferation from 10 to 14 weeks, hematopoietic phase at 15-18 weeks and reactivation to 22 weeks. This suggests that epithelial cell proliferation appears to take a backstage during the hematopoietic phase but undergo a second wave of progenitor cell proliferation with resurgence with termination of the hematopoietic phase. This was accompanied by increase in albumin and CYP450 gene expression compared to the 10 week fetal liver validating the maturation of fetal liver at this stage.

Week 10-11 Specification phase - genes at this stage were mostly embryonic stem cell factors and would be useful for in vitro IPSC manipulation. Week 14-15: Expansion / differentiation phase - Genes at this stage were extracellular matrix proteins Collagen, Laminin and Hyaluronic acid. Week 17-19: Hematopoiesis and Proliferation Week 20-24: Maturation, EMT phase - genes that were upregulated at this stage were FGF, FGFR and CTGF. Adult Liver: functional. Specifically, week 11 to 14 would represent the phase of hepatoblast playing the role of a transit-amplifying cell in expansion and differentiation into hepatic lineage. Similarly, week 19 to 22 would represent the phase of progenitor cell proliferation by mesenchymal epithelial transition. Week 23 to adult would represent the phase of hepatocyte maturation.

We have also identified key genes in each phase and are classified under transcriptional factors (GATA4, FOXA2 and CEBPa), growth factors (FGFR and CTGF) and extracellular matrix (COL12A1, LAMA3, Fibronectin and Hyurolic Acid). The progenitor liver cells were transplanted into SCID mice treated with thioacetamide and the degree of repopulation was analyzed. In

vivo transplantation of these cells in mice livers showed positive correlation of human cells engraftment.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AC133-Prominin mouse like1

AFP-Alpha Feta Protein

ALB-Albumin

APCS-Amyloid p component seurm

APOC2-Apolipoprotein C2

BEC- Billiary epithelial cells

BMP-Bone morphogenic protein

CEBPα-CCAAT/enhancer binding protein, alpha

CK19- Cytokeratin 19

COL12A1- Collagen, Type XII, Alpha 1-Like

CTGF- Connective tissue growth factor

CYP- Cytochrome P450

DAPI: 4',6-diamidino-2-phenylindol

Dex- Dexamethasone

DLK1- Protein delta homolog 1

DMEM: Dulbecco's Modified Eagle Medium

ECM-Extracellular matrix

EGF- Epithelial Growth factor

ELISA- Enzyme-linked immunosorbent assay

EMT-Epithelial-mesenchymal transition

EpCAM-Epithelial cell adhesion molecule

FBS: Fetal bovine serum

FGF -Fibroblast growth factor receptor

FOXA2 - Forkhead Box protein A2

GATA4-Globin transcription factor 4

GLI- Glioma-Associated Oncogene Homolog 1

HGF-Heptocyte growth factor

HLX- H2.0-Like Homeobox

Hnf3β- Hepatocyte Nuclear Factor 3,beta

HPX -Hemopexin

IF: Immunofluorescence

IGF1-Insulin Growth factor 1

IHC: Immunohistochemistry

IL-6-Interleukin-6

KLF4-Kruppel like factor4

LAMA3-Laminin-3

LETF- liver-enriched transcription factors

MAPK- Mitogen-Activated Protein Kinase 1

MEF- Mouse embryonic fibroblasts

MET- mesenchymal epithelial transition

NANOG- Nanog Homeobox

NODAL-Nodal growth differentiation factor

OCT4-Octamer binding transcription factor-4

ORM1-Orosomucoid 1

OSM- Oncostatin M

PBS: Phosphate-buffered saline

PDX1-pancreatic duodenal homrobox1

PROX1-Prodpero homeobox 1

RT-PCR: Reverse transcription-polymerase chain reaction

SERPINA1- Serpin peptidase inhibitor1

SHH-Sonic Hedgehog

SMA-Smooth Muscle Actin

SOX-Sex determining region

STAT3- Signal transducer and activator of transcription3

T Brachyury- T, Brachyury Homolog

TAT-Tyrosine aminotransferase

TGF-β-Transforming growth factor, beta

TSIX- Transcript, XIST Antisense RNA

VEGF-Vascular endothelial growth factor

WNT - Wingless-Type MMTV Integration Site Family, Member

XIST- X Inactive Specific Transcript

Chapter 1

Introduction

1.1 Background

The liver is a complex organ constituted of cells, such as hepatocytes, hepatocyte precursor cells (Scadden, 2006), stellate cells, kupffer cells, epithelial cells, sinusoidal epithelial cells, biliary epithelial cells, and fibroblasts(Kmiec, 2001). Hepatocytes make up to about 80% of the liver mass and are the primary functional cells in the liver. The cells are granular, protoplasmic; contain glycogen, fat or an iron compound. It is an important organ in our body that does physiological functions including metabolic reactions, energy storage, serum protein production, bile secretion, and defense against pathogenic infections. Liver disease is spread worldwide. The World Health Organization (WHO) estimated in 2013, death by liver cancer was increased to 50 million per year over last 2 decades. It was also reported that approximately1.3 million deaths worldwide are due to chronic viral hepatitis. It was reported that hepatic failure alone, reports for 1-2 million deaths per annum, and the main causes including: excessive alcohol consumption, aggressive forms of fatty liver disease, fibrosis, inflammatory liver conditions and unregulated ingestion of common over-the-counter medication, such as acetaminophen (Tynelol) (Rozga, 2006).

Although the liver disease has significant morbidity and mortality worldwide, medical advancement so far allows successful treatment for resection and transplantation surgeries only (Schwartz, 2014). This is an invasive procedure and is restricted by the availability of donor organs. Less than 23% of patients are on a waiting list to receive a transplant (Bellamy et al., 2001, Cowling et al., 2004).

Although Liver transplantation is currently the only option for treating both acute hepatic failure or end stage liver disease, a major and serious limitation is donor shortage. Recent advancement has turned its attention on identifying alternative mode of management for fatal liver disorders with regenerative medicine. Embryonic stem cells (ESC) provided alternative solution offering potential source of human hepatocytes. However, the limitation of using ESCs is that the production of large quantities of homogenous cells/tissue for clinical application. It has been well documented that there could be potential complications associated with the animal feeder layers on which human ES cells tend to rely on in addition to the risk of teratomas. Therefore the use of ESC remains a distant source for clinical application. The breakthrough techniques by Takahashi and Yamanaka for pluripotency induced in adult fibroblast gives an attractive alternative for ESC (Takahashi and Yamanaka, 2006). These induced pluripotent stem cells can be generated without ethical concerns, but their genome instability and low efficiency of cell production raises the same concerns as for ESCs when considering their clinical use.

Fetal liver progenitor cells have been identified and have been successfully differentiated into hepatocyte or bile epithelial cells (Rogler, 1997, Kubota and Reid, 2000, Dan et al., 2006) Kubota and Reid 2000). Previous studies have demonstrated in rat models that fetal hepatic stem/progenitor cells exhibited potency for reconstitution of adult liver but only under a specific set of conditions (Oertel et al., 2008). It is still unclear whether fetal liver progenitor cells, can reconstitute recipient livers not subjected to genetic modification. Its been reported that expansion of hepatocytes were compromised by phenotypic changes and karyotypic abnormalities over

prolonged culture durations (Delgado et al., 2005).

Hepatocyte transplantation is believed to be a potential solution to this problem. Human hepatocytes are in great demand for different purposes in translational research. In clinical applications, they could potentially be useful when transplanted directly into patients with liver failure or genetic disorders (Fox et al., 1998) or when employed in bioartificial liver devices for liver dialysis (Sussman et al., 1994). Although liver transplant is curative for many of these diseases, the complexity of the surgical procedure and shortage of healthy donor graft has limited the ability to transplant most patients with liver diseases. In fact, given the endemicity of Hepatitis B in Asia and epidemic of Hepatitis C in Western countries, the waiting list for transplant has grown exponentially and more patients are waiting longer on the list and dying before they can receive a transplant.

In addition, large numbers of human hepatocytes are needed for toxicology studies for drug development in the pharmaceutical industry. Testing of absorption, distribution, metabolism and excretion as well as toxicity in liver are considered as critical steps in new drug development. The use of reliable ex-vivo hepatocytes will allow rapid testing of drugs and reduce the need for exposing humans to phase 1 trial if hepatotoxicity can be detected earlier.

The major hurdles that have limited the use of human hepatocytes, are the lack of a continual, reliable source of cells and the technical difficulty involved in maintaining their differentiated hepatocytic functions *in vitro* for significant period of time (Leffert et al., 1978). Following liver injury, the human hepatocyte possesses tremendous intrinsic ability to proliferate and

regenerate itself *in vivo* (Fausto, 2004). However, despite the rapid advances in understanding the liver anatomy and physiology, it has not been possible to proliferate human hepatocytes reliably in culture or prevent them from dedifferentiation and losing their hepatocytic functions.

As a result of these limitations, there have been significant efforts to identify and utilize alternative sources of cells that can be expanded easily in culture, and subsequently manipulated to give rise to hepatocytes, either by directed differentiation (hepatocyte linage stem/ progenitor cells) or transdifferentiation (stem cells from other lineages)(Dan and Yeoh, 2008). Candidate cells that have been reported to have this potential includes embryonic stem cells, hematopoietic stem cells, mesenchymal stem cells (MSC) or mesenchymal lineage stem cells, fetal liver progenitor cells, adult liver progenitor cells or more recently, induced pluripotent stem cell or reprogrammed somatic cell(Dan and Yeoh, 2008).

The knowledge gained from the exploration of these steps from lab to bedside is important in attempting to use these cells for therapeutic application. We believe both these cell types are easier to convert, as hepatocytes are amenable to scale up cultures to provide large numbers of hepatocytes in a safe, efficient way in comparison with mesenchymal stromal cells

Limitation in past studies includes: 1. No systematic temporal expression studied on human fetal liver developmental stages and its regulatory mechanisms. 2. Our gaps in knowledge about the liver maturation. 3. Understanding the microenvironment during development helps to close the gap in culture system. Using this knowledge could bring us closer to liver

disease treatment in a promising way. In this study we have focused on temporal expression of human fetal liver development during the second trimester. We hypothesize that the fetal compared to adult liver environment, would shed light on the key maturation signals that drive fetal liver progenitor cell proliferation and differentiation.

1.2 Literature review of previous studies on liver development in animal models

Liver development is a complex process of distinct biological events. Each stage of differentiation is controlled by mechanisms in addition to the extracellular signals. During the last decade, substantial progress has been made in understanding the molecular mechanisms that direct early aspects of mammalian liver development. Studies using tissue explant cultures and molecular biology methods in addition to the analysis of the transgenic and knockout mice have identified signaling molecules and transcription factors which are required for the establishment of hepatogenesis.

1.3 Hepatic lineage Specification

During gastrulation, liver is derived from the endoderm, one of the three germ layers. The endoderm defines the primitive gut and gives rise to the epithelial compartment of the gastrointestinal tract. The endoderm also gives rise to other organs including the pancreas and the thyroid gland (Lemaigre, 2009). Fate mapping studies in mice where endodermal cells were labeled with a dye showed that liver progenitor cells originate from three endodermal domains: two domains are paired and located laterally, and the third domain is found along the ventral midline (Tremblay and Zaret, 2005, Zaret, 2008).

Experiments based on tissue explants and genetically engineered animal model have revealed a number of important mechanisms underlying liver specification (Zaret, 2002, Lemaigre and Zaret, 2004).

1.3.1 Specifying the hepatic lineage by cardiac mesoderm

A crucial question in the area of the gut organogenesis is how individual tissues are specified at different domains along the antero-posterior axis of the endoderm. Studies in model organisms have shown that endodermal domains are usually patterned by interactions with overlying mesodermal tissue (Douarin, 1975). The classical tissue transplantation studies performed by LeDouarin, using chick embryos, showed that cardiogenic mesoderm, which is transiently in the close vicinity of the prospective hepatic endoderm, provides a signal that is crucial for inducing liver progenitors in endoderm (Douarin, 1975).

On the other side mesoderm from other areas of the chick embryo does not induce the liver. Interestingly, at an earlier stage, the endoderm itself is important for inducing cardiogenic mesoderm (Sugi and Lough, 1995). Fibroblast growth factors (Fgfs) signaling from the cardiac mesoderm induces the liver in the ventral foregut endoderm. At the time of hepatogenesis, the cardiogenic mesoderm express at least three out of the eighteen known Fgfs, and the ventral foregut endoderm expresses at least two of the four tyrosine kinase Fgf receptors (Jung et al., 1999). At the time of hepatic induction, the endoderm uniquely expresses Fgf receptor 4 (Fgfr4) (Stark et al., 1991) and both the endoderm and cardiac mesoderm express Fgf receptor 1 (Fgfr1) (Sugi and Lough, 1995). In situ immunohistiochemistry studies showed that

Fgf1 and Fgf2 are both induced in the cardiac mesoderm at the 7-8 somite stages in mouse(Jung et al., 1999). Purified Fgf1 and Fgf2 were each found to efficiently induce early liver-specific genes within the ventral foregut endoderm, when the endoderm was isolated from 2-6 somite stages. The induction of serum albumin (Alb), α-fetoprotein (Afp), and transthyretin (Ttr), was as strong as that induced by cardiac mesoderm. In contrast, Fgf8 had only partial hepatogenic activity and it failed to induce hepatic development. However, it was found to contribute toward the morphogenic outgrowth of the hepatic tissue following specification. The early expression of Fgf8 or a related molecule appears to strengthen the morphogenetic activity of the emerging hepatic cells. It appears, that Fgf8 works together with a signal that has not been identified to stimulate cell outgrowth (Jung et al., 1999).

The isolated ventral foregut endoderm does not remain undifferentiated when it is cultivated without cardiogenic mesoderm or Fgfs. Rather; it starts the expression of pancreatic genes, as a default lineage (Deutsch et al., 2001). Fgf or cardiogenic mesoderm suppress the pancreatic program in the endoderm and induces the liver program. So ventral foregut endoderm consist of a multipotential cell population that undergoes a cell-fate choice during spatial patterning. The ventral pancreatic bud derives from the most distal endoderm that extends away from the developing heart and is able to follow the default pancreaticfate.

1.3.2 Specifying the hepatic lineage by septum transversum mesenchyme

The early chick studies of LeDouarin identified a second stage of hepatic induction, which appears when mesoderm derived cells in the septum transversum promote growth and further differentiation of the newly specified hepatic endoderm (Douarin, 1975, Fukuda-Taira, 1981).

These additional hepatogenic signals originate from septum transversum mesenchyme (STM) cells. The septum transversum derives from lateral plate mesoderm and gives rise to the epicardium of the heart and also the diaphragm. Before hepatic induction, prospective septum transversum mesenchyme cells surround the developing cardiac re- gion near the ventral foregut endoderm. Bone morphogenetic proteins 2 and 4 (Bmp2 and Bmp4) are strongly expressed in the STM, before and during hepatic induction.

(Rossi et al., 2001) confirmed that STM collaborate with developing cardiac tissue to control specification of the liver lineage. The role of Bmp4 signaling during the onset of hepatogenesis has been confirmed by experiments with Noggin (Bmp4 antagonist). This molecule was found to inhibit albumin mRNA expression in co-culture of cardiac tissue and 2-6 somite stage ventral endoderm. This result was contradictory with previous finding by Jung et al. in that FGF alone was sufficient to induce hepatogenesis within cultured ventral endoderm. However, the endoderm cultures contained small numbers of Mrg1 positive cells, which is a marker of STM. The amount of STM cells was sufficient to supply a sufficient amount of Bmps to allow hepatic induction by exogenously added FGFs (Rossi et al., 2001). Additionally, for the induction of hepatogenesis, secretion of Bmps by STM appears to be

critical for outgrowth of the budding hepatoblasts. The Fgfs and Bmps act in a concert manner on the ventral foregut endoderm to direct the onset of hepatogenesis.

The *Hlx* knockout embryos additionally confirmed the STM role in controlling developmental growth of the liver. *Hlx* gene encodes a homeobox transcription factor and its expression in developing liver is restricted to cells derived from STM. Mouse embryos lacking the *Hlx* gene start the liver development normally, however, by E15.5 the mutant livers had failed to expand and reached only 3% of the size of control livers (Hentsch et al., 1996). Although the targets of *Hlx* are not known, it must be required for expression of paracrine factors, from STM, that control hepatogenesis (such as hepatocyte growth factor (HGF)).

1.3.3 Hepatic gene induction in embryonic endoderm cells

The tissue interactions described the exact location and timing of hepatogenesis during embryonic development. At least three different kinds of mesoderm cells, including the cardiac mesoderm (Douarin, 1975, Gualdi et al., 1996), the septum transversum mesenchyme (STM) (Rossi et al., 2001), end endothelial cells (Cleaver and Melton, 2003), coordinately induce liver development in the endoderm, apparently by employing different signaling molecules (Zaret, 2002, Duncan, 2003b). Nevertheless, the mechanisms by which the cells of the endoderm really follow and adopt a hepatic fate are best considered as intracellular responses to these signals. The intracellular network by which FGF signaling helps induce hepatic genes and stabilize

emerging hepatic cells within the endodermal epithelium has just recently been elucidated.

FGF-mediated induction of hepatic genes function through the MAPK pathway and not the PI3K/AKT pathway. Although the PI3K/AKT pathway is activated in foregut endoderm cells, its inhibition does not block hepatic gene induction in explants; however it does block tissue growth. At the beginning of hepatogenesis, the FGF/MAPK and PI3K/AKT pathways are induced separately in the foregut endoderm and do not cross- regulate at the initial stages of tissue patterning (Calmont et al., 2006). The inherent ability of FGF to activate MAPK and not PI3K in the foregut endoderm helps define the intracellular network that give the endoderm cells the ability to induce liver and explain how a common signal such as FGF trigger a specific cellular response. There is a strong correlation between the expression of different FGF ligands and phospho-ERK activation during the period of hepatic specification. Although FGFs represent a proliferative signal for the endoderm shortly after tissue patterning (Bhushan et al., 2001)and MAPK signaling can stimulate cell proliferation (Lavine et al., 2005). Calmont *et al*. demonstrated that FGF/MAPK signaling initiates the hepatic differentiation and that it is distinct from the effects on cell proliferation and growth.

1.3.4 Growth of hepatic endoderm into the liver bud

The liver emerges from the definitive gut endoderm first as a thickening of the ventral endoderm epithelium and then as a bud of cells that proliferates and migrates into the surrounding septum transversum mesenchyme. The septum transversum is a collagen-rich environment colonized by loosely

joined mesenchyme cells (Cascio and Zaret, 1991), which defines the area of the embryonic body cavity into which the hepatic bud grows. These pre-hepatic cells, which delaminate from the foregut and migrate into septum transversum are called hepatoblasts (Medlock and Haar, 1983). Their analyses in culture suggest that they are bipotential, capable of giving rise to both the hepatocyte and cholangiocyte cell lineages (Rogler, 1997). As the hepatoblasts migrate they closely associate with primitive sinusoidal endothelial cells that form capillary-like structure between the migrating hepatic strings (Enzan et al., 1997). The hepatoblast have irregular shape, large nuclear to cytoplasmic ratio and relatively few organelles when compared to mature hepatocytes. The process of differentiation of hepatoblast to hepatocyte is gradual, taking several days during development of the rodent embryo.

After the liver bud is generated, it appears that other groups of growth signaling pathways are involved in further liver specification. These signals are, hepatocyte growth factor (HGF), oncostatin M (OSM) and glucocorticoids.

Hepatocyte growth factor (HGF) is a powerful mitogen originally discovered to play a role in the regeneration of the adult liver, after partial hepatectomy (Michalopoulos and DeFrances, 1997). The HGF/c-Met pathway mediates the interaction between mesenchyme and epithelial cells during development since HGF is expressed in the STM that surrounds the developing liver bud, and c-met, the HGF receptor, is expressed on embryonic hepatocytes (Schmidt et al., 1995). Mice lacking HGF fail to complete development and

die in uterus. The mutation affects the embryonic liver, which is reduced in size and shows extensive loss of parenchymal cells. In addition, development of the placenta, mainly of trophoblast cells, is impaired. Thus, HGF is essential for the development of several epithelial organs (Schmidt et al., 1995).

Hematopoiesis plays an important role in hepatic maturation. After the liver bud emerges from the gut tube, hematopoietic cells migrate there and propagate. The hematopoietic cells produce oncostatin M (OSM), a growth factor belonging to the interleukin-6 (IL-6) family (Zarling et al., 1986). In the developing liver, OSM is ex- pressed by CD45+ hematopoietic cells, but not by hepatocytes. OSM stimulates the expression of hepatic differentiation markers and induces morphologic changes and multiple liver-specific functions like ammonia clearance, lipid synthesis, glycogen synthesis, detoxification, and cell adhesion. With the maturation of bone marrow and spleen around birth, hematopoiesis in the liver reduces and hematopoietic stem cells migrate from the liver to the organs responsible for adult-type hematopoiesis (Kinoshita and Miyajima, 2002). While OSM expression in the liver starts in mid gestation and de- creases in postnatal stages, HGF is mainly expressed in the liver during the first few days after birth. OSM and HGF induce hepatic maturation through different signaling path- ways. Hepatic maturation induced by OSM depends on STAT3 and HGF-induced differentiation is STAT3-independent. Like OSM, HGF in the presence of dexamethasone induced expression of glucose-6-phosphatase (G6P), tyrosine amino transferase (TAT), and accumulation of glycogen in fetal hepatic cells, but at a lower level than OSM. Both OSM and HGF induce production of

albumin (ALB) but its secretion appears only in response to OSM (Kamiya et al., 2001).

Glucocorticoids are involved in hepatic maturation and alter the proliferation and function of adult hepatocytes. In the fetal liver, physiological concentration of dexamethasone (Dex), a synthetic glucocorticoid, suppress α-fetoprotein (AFP) production and DNA synthesis and promote albumin (ALB) production. OSM alone fails to induce differentiated liver phenotypes, implying the importance of glucocorticoids as triggers for hepatic maturation (Kinoshita and Miyajima, 2002).

1.3.5 Other molecules affecting hepatogenesis

Other signaling molecule affecting the developing hepatocytes is transforming growth factor β (TGFβ). Liver development was found to be severely disturbed in Smad2+/− and Smad3+/− mouse embryos at E14.5 (Weinstein et al., 2001). The presence and composition of extracellular matrix (ECM) has a significant effect on the gene expression profiles of cultured primary hepatocytes (Michalopoulos et al., 2001). A role of ECM signaling during the development of the liver emanate from studies of β-1 integrin. Chimeric mice generated by combining wild type embryos with β-1 integrin−/− ES cells showed that cells lacking β-1 integrin were unable to colonize the liver. Hence, there is a requirement for β-1 integrin in defining or maintaining the hepatocyte cell lineage (Fassler and Meyer, 1995).

1.4 Transcriptional regulation of early hepatogenesis

The creation of the liver occurs in a two-step process, beginning with the establishment of competence in the foregut endoderm to respond to signals

from cardiac mesoderm, followed by the induction of liver-specific gene expression.

A main question in gut organogenesis is if there are present different domains of developmental competence in the endoderm that decided where different tissues could arise. Such a domain competence could be due to differential expression of transcription factors or signal-transduction molecules along the antero-posterior axis in the endoderm. There is some evidence for a domain of competence for liver formation and there is significant importance of transcription factors in establishing the endodermal domain that gives rise to liver. Three highly related FoxA (forkhead box A) proteins, are expressed in the fetal and adult liver as well as other endoderm-derived tissues. The FoxA pro- teins regulate almost all liver-specific genes as well as genes in the lung and pancreas (Zaret, 1999). The FOXA (HNF3) proteins were first discovered by their ability to bind to the promoters of the genes encoding α-1-antytrypsin (A1AT) and transthyretin (TTR) (Kaestner et al., 1999). In the endoderm, the beginning of FoxA gene expression precedes the induction of the hepatic program by FGFs signals. Expression of FoxA2 (formerly Hnf3β) starts in the primitive streak during gastrulation. FoxA1 (Hnf3α) (Sasaki and Hogan, 1993) expression initiates in the gut endoderm at E7-E8, before organogenesis, whereas FoxA3 (Hnf3γ) expression begins in the gut endoderm at E8-E9 but is restricted to the midgut and hindgut regions (Ang et al., 1993, Monaghan et al., 1993). FoxA1 and FoxA3 genes are also expressed in early neural tissues (ectoderm) and in the notochord (mesoderm), but all FoxA genes are restricted to the endoderm-derived organs in adults (Lai et al., 1991).

Among the transcription factors found to be expressed in the definitive endoderm, the transcription factors Hnf3β and Gata4 have each been found to act as mediators of competence in the foregut endoderm (Kaestner et al., 1999). In vivo footprinting analyses of the albumin enhancer in E9.5 mouse liver buds revealed that several binding sites, including Hnf3β, Gata4 and Nf-1 sites, were occupied. In contrast, extracts from the gut endoderm, a tissue capable to follow a hepatic fate but uncommitted and not expressing albumin, showed in this same assay that only Hnf3β and Gata4 sites were occupied (Gualdi et al., 1996, Bossard and Zaret, 1998). Hnf3β and Gata4 are able to bind silent hepatic enhancers and mark them as possessing the potential to be expressed following induction.

Other transcription factors involved in early stages of liver development are Hex and Prox1. Hex encodes a homeobox transcription factor; which is essential for very early aspects of hepatic development and which is amongst the earliest markers of developing liver. Hex mRNA at E8.5 is restricted to two distinct regions within the ventral endoderm, the future sites of the liver and thyroid (Keng et al., 1998). Knockout experiments have also revealed a role of the second homeobox transcription factor called Prox1 during early stages of hepatic development (Oliver et al., 1993).

1.4.1 Transcriptional regulation of hepatic maturation

During the 1980s a huge effort by many labs allowed the identification of transcription factors that bound transcriptional regulatory elements of genes that are predominantly expressed in the liver (Lai et al., 1991). Among liver-specific transcription factors possessing various structural motives are homeodomain proteins HNF1α and HNF1β, the winged helix proteins

FOXA1, FOXA2, and FOXA3, the leucine zipper proteins C/EBPα and β, the orphan nuclear receptor HNF4α and the onecut protein HNF6. Disturbance of some genes does have serious consequences on liver function. Additionally, although fetal livers develop normally, hepatocyte proliferation was decreased in newborn c/ebpα−/− livers (Timchenko et al., 1997). Transcription factors cooperate to coordinate gene expression so many mutations do not disrupt hepatocyte differentiation. The majority of promoters are bound by multiple factors and, therefore, it seems that loss of a specific factor can be compensated for by other transcription factors present within the cell. As this model may be generally applicable it has been confirmed that during development of the fetal liver the nuclear hormone receptor, Hnf4α, is crucial for expression of a large array of genes that define hepatocyte function (Li et al., 2000). Hnf4α acts as an essential regulator of hepatocyte differentiation. Hnf1α transcriptional regulatory elements possess the Hnf4α binding site. Hnf4α controls hepatocyte differentiation through the activation of a cascade of transcription factors that eventually define the gene expression profile of the mature hepatocytes. Hnf4α−/− embryos arrest during gastrulation, prior the start of hepatogenesis, because of defects in visceral endoderm function (Duncan et al., 1997). The combined application of molecular genetics, molecular biology and embryology led us to slowly understand of the mechanisms that control hepatogenesis.

1.5 Hepatogenesis in vitro

The microenvironment of establishing hepatocytes is a consistently modifying process of successively occurring biological events (Duncan, 2003a). Every stage of cell development and differentiation is firmly

regulated by intra and extracellular contact along with cell independent mechanisms. Liver development is controlled by several distinctive paracrine factors. The critical role was assigned to transcription factors and cytokines, which have been documented as important molecules during the key steps of the liver development. NODAL (Activin A), FGFs, BMP, HGF and OSM are herein the most essential extracellular signals (Borrello et al., 1992, Duncan, 2000, 2003a). At the intracellular stage, the liver enriched transcription factors like hepatocyte nuclear factor ((HNF)3α, β), HNF4α, HNF1α, β, HNF6, and CCAAT enhancer binding protein ((C/EBP)α, β) act at particular developmental phases in order to control liver-specific gene expression (Kyrmizi et al., 2006).

In theory, experimental conditions have been applied to induce cultured pluripotent human ES cells into functional hepatocytes, are based on reconstructing the *in vivo* microenvironment. They used the supplement of soluble medium factors and reconstruction of cell-matrix and cell-cell interactions. Overexpression of liver-enriched transcription factors (LETF) genes can be an alternative, but has the limitations.

1.6 Induction of hepatic cell fate via supplement of soluble factors cytokines, growth factors, hormones)

The use of growth factors and cytokines is essential for human ES cells differentiation into hepatocyte-like cell *in vitro*. Hormones and corticosteroids relatively perform a supporting role.

The development of endoderm shows an important part of initiating the early stages of liver development. Two situations are required to stimulate

approximately 70- 80% of definitive endoderm from human ES cells: signaling by Activin A/NODAL ligands and release from inhibitory signals produced by PI3K through insulin (D'Amour et al., 2005). Primarily, Activin A enriches human ES cell culture for definitive endoderm. FGFs and BMPs, are effective in mediating early hepatic differentiation. HGF supports mid-late hepatic phenotype (e.g. ALB expression) (Kumashiro et al., 2005) but do not induce functional maturation. Stepwise supplement of FGF, HGF and a combination of insulin-transferrin-sodium selenite (ITS), dexamethasone and OSM was successful. Additional variations of the last consecutive approach also result in 70-80% purity of ES-derived hepatocytes within the culture system(Agarwal et al., 2008). Inseparable aspect of most differentiation protocols is the co-exposure to serum, which includes hormones, growth factors and other undefined substances that will influence the stochastic differentiation of pluripotent ES cells (Jochheim et al., 2004). Recently many efforts have been made to function under serum-free conditions (Hay et al., 2008a,b).

1.7 Hypothesis

No studies have yet to classify and characterize the genetic and phenotypic mechanisms in developing human fetal liver. The unique access for us to isolate the human fetal liver has given an edge over to study the insights of the developing human fetal liver. We can identify the key regulatory mechanism that regulate during the liver development.

By identifying the key transcriptional factors, growth factors and extra cellular matrix in developing human fetal liver we can manipulate them in

vitro for ideal cell culture for differentiating the functional hepatocytes. The ability to understand the human fetal development gives clues to maintain and expand the cells in vitro and control differentiation. These cells can be further transplanted into the cirrhotic liver for principle proof of survival

1.8 Aim of the study

To understand the key regulatory mechanisms of developing human fetal liver during mid-gestation period of organogenesis

Sub aim

1. To determine the regulatory factors that control epithelial and hepatocytic specification during development.
    a. To determine the regulatory mechanisms of developing fetal liver.
    b. To determine the key transcriptional factor, growth factor and extracellular matrix.
2. Establishment of *in vitro* culture conditions for hepatic differentiation and maturation.
    a) To identify the key growth factors for optimal cell culture for efficiency of expansion
    b) To optimize the Extracellular matrix for ideal hepatocyte culture condition.
3. Proof of survival efficacy in small and large animal models in acute liver failure
    c) Cellular transplant in mice model of acute liver failure and liver cirrhosis

Chapter 2

Phases in the development of human fetal liver

2.1 Background

The core concept of this whole study is to explore the genetic regulation during the development of human fetal liver at second trimester. In this chapter we mainly describe about the characterization of the developing human fetal liver at different gestational stages at second trimester with known liver and stem cell specific gene set using PCR array and microarray analysis. This chapter also validates the previous studies on fetal liver development and also gives us the new understanding about the classification of liver development and its phases, which can be used in *in vitro* applications. This key information can be used in to enhance the differentiation and maturation of hepatocytes in vitro.

During gastrulation, the endoderm germ layer is formed, which is further divided into foregut, midgut and hindgut regions. In recent studies using fate-mapping technique in mouse embryo it was proved that liver originates from the ventral foregut endoderm (Tremblay and Zaret, 2005). The fetal liver develops from the foregut endoderm, which the stem cells specify into liver lineage as the liver bud is formed at D20 of gestation, induced by FGF2 and BMP4 from the cardiac mesoderm (Rossi et al., 2001). Hepatic diverticulum is divided into two regions; anterior portion, which give rise to liver, cells and posterior portion give rise to extra hepatic cells. The cells derived after lineage differentiation to the liver bud are called bipotential hepatoblast cells during development (Tanaka et al., 2009). These bipotential hepatoblasts undergo rapid expansion by eight weeks of gestation, and then they differentiate into hepatoblasts or biliary cells. During the liver bud stage, the liver development undergoes rapid growth and colonized by hematopoietic

cells to become the major fetal hematopoietic organ (Christensen et al., 2004). This hepatocytic development process has been well studied in mice, which shows that, at this bipotential stage hepatoblast undergoes specification from ED9.5 to 13 and hepatoblast differentiation from ED 14 to hepatocytes (Lemaigre, 2003). To further complicate issues, the fetal liver is also the site of hematopoiesis at embryonic E7 (Gallacher et al., 2000).

However, to our knowledge, there are very few systematic serial studies of developing human fetal liver on human fetal liver with both genomic and phenotypic analyses. The gene expression changes in this second trimester study will provide us knowledge for manipulating the microenvironment or reprogramming non-hepatic cells to give rise to functional liver cells *in vitro*.

In order to determine the systematic development of human fetal liver during second trimester, and to identify the key genes that are regulated, we characterized the gene expression changes at the following gestational stages namely 10 weeks (10 to 11 weeks), 14 weeks (14 to 17 weeks), 18 weeks (17 to 19 weeks), 22 weeks (21 to 24 weeks) by both full-genome microarray analysis and next generation RNA sequencing. The comparison was made with the human adult liver. In addition, to validate this study, qRT-PCR array was performed for 364 known genes expressed in the liver. To understand the phenotypic changes, the temporal expression pattern was examined by immunohistochemistry and histology.

2.2 Materials and Methods

2.2.1 Source and isolation of human fetal liver cells

Human fetal livers were obtained from the KK women and children's hospital Singapore and at the National University Hospital of Singapore in accordance to the protocol approved by the institutional review board of both National University of Singapore and KK hospital. Written informed consent from the donor or the next of kin was obtained for use of all samples used in this study. Fetal liver was freshly isolated and transported immediately to the laboratory for processing in DMEM basal medium (Life technologies Catalog number: 11965-092). Primary fetal liver cells were raised and kept in culture using the Fetal Hepatocyte medium (CSHFM) as previously described (Lazaro et al., 2003, Dan et al., 2006).

Table 1: Number of samples used in the study

Gestational age	Number	Experiment
10 weeks	3	NGS, Microarray, PCR array Immunofluorescence and FACS
14 weeks	3	NGS, Microarray, PCR array Immunofluorescence and FACS
17 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS
18 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS
19 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS
21 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS
22 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS

23 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS
Adult liver	5	NGS, Microarray, PCR array Immunofluorescence and FACS

The samples were selected based time frame of the collection and based on the quality of the RNA.

2.2.2 Immunohistochemistry and Immunofluorescence

All the liver tissue was processed fresh. Sections for Immunofluorescence were fixed with 10% cold formalin. Slides for Immunohistochemistry were fixed with 1:1 volume mixture of -20'C acetone followed by 0.3% H_2O_2 to block endogenous peroxides. Sections were then blocked with appropriate serum 10% for 30 minutes. For immunohistochemistry, the slides were de-paraffinized by immersing them zs1in 3 changes of Xylene for 5min each. This was followed by immersing the slides in a graded series of ethanol and finally washed thoroughly in deionized water. For antigen retrieval, tissues were incubated with Antigen retrival solution (Dako) for 20min at 37oC. The sections were placed in a jar containing DAB solution and incubated until a brown color developed.

Immunofluorescence methodology: Formalin Fixed frozen tissue sections were washed in PBS and blocked with 5% normal goat serum at room temperature for 1h. A primary antibody cocktail (1:100) was prepared in PBS. Tissue sections were incubated with the antibody overnight at 4^oC. The following day, tissue sections were washed in PBS and incubated with a secondary antibody cocktail of Alexa Fluor® 488 (1:1000) or Alexa Fluor®

594 (1:1000) for 1h. After this, the sections were washed in PBS and incubated with DAPI (1:2000) to stain the nucleus. The sections were washed with PBS and mounted in fluorescent mounting medium. The slides were protected from light and later viewed under a confocal laser-scanning microscope (Model Fluoview FV- 1000, Olympus Optical Co., Tokyo, Japan). Positive cells was determined by manual counting of positive immunofluorescent cells to DAPI positive nucleus in 3 separate random fields (at 40X magnification objective) on the Olympus microscope.

Primary antibodies against human antigens were applied to slides for overnight at 4 degrees and they include anti-albumin (ICN Pharmaceuticals, Aurora, OH), α-fetoprotein, vimentin, smooth muscle actin (Dako Cytomation, Inc.,Carpinteria, CA), CD44, CD133 (abcam), CD45 (biolegend) EPCAM (novus ResearchProducts, littleton, CO), CK19 (Amersham, Buckinghamshire, England). For Immunohistochemistry, Horseradishperoxidase–conjugated anti-mouse, anti-rabbit and anti-goat antibodies (all from Vector laboratories, Burlingame, CA) served as secondary antibodies with positive detection based on reaction with 3,3-diaminobenzidine tetra hydrochloride. Fluorescent detection was by Alexa Fluor® 488 or Alexa Fluor® 594 conjugated secondary antibodies (Invitrogen, Carlsbad,CA).

2.2.3 RNA isolation

Total RNA was isolated using Trizol reagent (Invitrogen, Austin, TX Catalog number: 15596-026), 10 week, 14week, 18 week, 22 week and human adult

liver. Isolated RNA was subjected to bioanalyser to check the integrity and used which was more than 8 in all our experiments.

Materials and instrument

Trizol reagent (Invitrogen, Austin, TX Catalog number: 15596-026) 70% ethanol β-mercaptoethanol Spectrophotometer (Nanodrop; Model No. ND1000, Thermo Scientific, MA, USA)

Procedure

HOMOGENIZATION: Clean Polytron homogenizer with RNzap, then wash with EtOH, dH2O and finally with 1 ml of Trizol reagent (every time run Polytron at full speed for about a minute). Add 500ul Trizol into a 5ml tube. Transfer freshly isolated liver tisues to an eppendorf tube, Add with Trizol (500ul), pool together to bring the volume to 1ml total.

Phase separation: Shake the homogenate for 10 minutes at RT to permit the complete dissociation of nucleoprotein complexes. Supplement the homogenate with 0.2ml of chloroform per 1 ml of Trizol, cover the samples tightly and shake vigorously (vortex) for 15 seconds. Incubate the mixture at RT for at least 10 minutes (up to 30 min). Centrifuge at 14, 000g for 15 minutes at 4^{o}C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 60% of the volume of Trizol used for homogenization**.** Add isopropanol and the high salt solution (0.8 M Sodium Citrate, 1.2 M NaCl),

each at one half volume of the aqueous phase. Keep samples at –20 oC O/N (or at least 1 h). Centrifuge at 14, 000g for 30 minutes at 4 oC.

RNA wash and solubilization: Discard the supernatant. Wash 2x with 1.0 ml of 75% ethanol per 1ml TRIzol used for the initial homogenization. Vortex, then spin down at 7, 500 rpm/5 minutes at RT. Remove ETOH. Let RNA pellet briefly dry. Dissolve in 20-30ul diH2O Depending on the amounts, the resultant total RNA may be subjected to further clean-up with Qiagen RNEasy columns. However, it is not recommended if we use get less than 10 ug of total RNA (expect 20-40 % loss from the clean-up). Quality controls using Agilent Bioanalyzer.

2.2.4Qualitative Real Time - PCR Array

We have used a set of known liver genes that were described in previous studies. We customised the PCR array with 364 genes in 4 different plates. These plates were classified as 1. liver and stem cell genes, 2. Growth factors and 3. Extracellular matrix. These customised plates were manufactured by sabiosciences.

Principle

Quantitative real time RT-PCR (qRTPCR) is used to amplify a certain region of cDNA using forward and reverse oligonucleotides (primers) specific to that region. The amplified double-stranded DNA is quantified by measuring the amount of fluorescence dye, such as SYBR green, that intercalates with the DNA.

Firstly, the two strands of DNA are separated to form two template strands by

heating at a high temperature for a brief period of time. This is followed by amplification process during which the primers bind to the template strands and the enzyme, taq DNA polymerase extends the specific region of template DNA. The primers are designed such that they are complementary only to one specific region of the DNA which represents a particular gene of interest. Each amplification cycle has alternating temperature to allow the sequential process of primer binding, extension and denaturation. After several cycles of amplification, adequate copies of cDNA are made.

During the amplification process, SYBR green fluorescence dye intercalates with double stranded DNA and the qRTPCR instrument quantifies the fluorescence intensity of the intercalated dye and provides a numerical value called the cycle threshold (Ct) value (Bustin 2000). The $2^{-[\Delta\Delta Ct]}$ method is used to calculate the relative expression of a particular gene in experimental groups when compared with control groups (Livak and Schmittgen 2001).

25mg of RNA were reverse transcribed (SABiosciences first strand kit Catolgue No - 330421), and PCR array was performed with 364 genes that were divided into four different 96 well plates. This was used to determine expression of several genes using specific primer pairs.

2.2.5 Illumina microarray analysis

Once we have classified the known set of liver genes, we then probed for the high throughput gene analysis.

Principle of microarray experiment

A microarray experiment is performed to identify gene expression changes on

a high-throughput basis. This procedure involves the basic principle of nucleic acid hybridization. A microarray chip is made up of thousands of oligonucleotides attached onto a solid surface. RNA is extracted from the sample to be profiled and converted to cDNA by reverse transcription. The cDNA is converted to cRNA by in vitro transcription with fluorescence or biotin tags. The biotin-labeled cRNA is spotted on the microarray chip for hybridization for a certain period of time and the non-hybridized cRNA is washed away. Finally, the microarray chip is scanned under laser light and the images obtained are analyzed (Heller 2002; Murphy 2002).

2.2.5.1 Sample amplification and hybridization

RNA (500ng) was amplified using the Illumina Total Prep RNA Amplification Kit, according to manufacturer's instructions. cRNA was hybridized to Illumina Human Sentrix-6 bead chips Ver.2, according to standard Illumina protocols (http://www.illumina.com).

2.2.5.2 Raw data curation and normalization of Illumina microarray

Chips scans were processed using Illumina BeadScan and BeadStudio software packages and summarized data was generated in BeadStudio (version 3.1). The summarized data from BeadStudio was imported into R/BioConductor using the read Bead function from the Bead Explorer package Background adjustment and quintile normalization was performed using algorithms within the Illumine package (function: bg. adjust and normalize. quintiles). The normalized data was exported out off R/BioConductor with write. BeadData function.

2.2.5.3 Normalization and statistical analysis of Illumina microarray data

All microarray experiments were conducted in biological triplicate. A gene was considered detected if the average detection score (p-value) of the three replicates was less than 0.05. Quintile normalization and statistic calculations were carried out using the Partek software.

2.2.6 Next generation sequencing data

Our analysis from PCR array and Microarray data showed different phases in liver development (Discussed later in the chapter). We need an advanced tool to analyze the important genetic differences at each stage. Although microarray provided us a large amount of data the limitation of using the denominator of genes greater than 2 fold restrict us to existing genomic sequencing information. RNA-seq experiments on the other hand work well for investigating both known transcripts and exploring new ones. Therefore, RNA-seq is ideal for discovery-based experiments. And while microarrays are limited to the reference information available during production, RNA-seq experiments may be updated as new sequence information is obtained.

Second, RNA-seq delivers low background signal. This is because DNA sequences can be unambiguously mapped to unique regions of the genome. As a result, noise in the experiment is easily eliminated during analysis. Hybridization issues seen with microarrays, such as cross-hybridization or non-ideal hybridization kinetics, are also eliminated in RNA-seq experiments. This offers another signal-to-noise advantage.

Finally, RNA-seq has the ability to quantify a large dynamic range of expression levels, with absolute rather than relative values. Even with

organisms lacking a reference genome, de novo transcriptome assembly and differential expression analysis can be performed. There is no upper limit for this quantification, and at Cofactor, we can help you to determine the number of reads you need to find the information you're looking for with high levels of reproducibility between both technical and biological replicates.

For the next generation RNA sequencing data, we isolated RNA from the tissue samples determined the RIN number and passed on to our collaborator in Genome Institute of Singapore for the sequencing.

Results:

2.3 Liver architecture – Hepatic portal vein

The light microscopic images of H&E show the cellular architecture of liver.

In our observation of H&E on the human fetal liver, the ductal plate appeared small and distinctly differentiable from the late stages at 14w and became more organized with portal mesenchyme during 18w. We notice that by 22 weeks, portal triads are formed and are surrounded by hepatocytes, which are arranged in single cell sheets known as hepatic plates, separated by sinusoid spaces that are connected to a network of blood vessels (capillaries) (**Figure 2.1**).

2.4 Phenotypic characterization

To determine the different types of cells present in the developing human liver, we characterized the phenotypic pattern at each stage with Immunohistochemistry using different markers namely Epithelial cell adhesion molecule (EpCAM), Vimentin, Smooth muscle actin (SMA), Alpha

feta protein (AFP), CD133 (PROMININ 1), CD45, Albumin and cytokeratin-19 (CK-19) on each time points. These markers are classified in different cell types and play vital functions during the fetal liver development.

Table 2: Markers involved in fetal liver development

EpCAM	Epithelial stem cell
CD133	Stem cell marker
CD44	Stem cell marker
CD45	Hematopoietic stem cell marker
Albumin	Hepatocyte marker
AFP	Fetal hepatoblast marker
Vimentin	Mesenchymal marker
SMA	myofibroblast marker
CK19	Liver progenitor cell

2.4.1 EpCAM

EpCAM is a cell-surface glycoprotein expressed on cholangiocytes, but the expression on matured hepatocytes is diminished in adult liver (de Boer et al., 1999). It was also reported that multipotent progenitor cells derived from human fetal liver expressing EpCAM (Dan et al., 2006). EpCAM also serves as a useful marker to track the hepatic differentiation, as it is expressed in the progenitor cells cytoplasm and is lost in the adult hepatocytes (Rao et al., 2008).

The expression profile of EpCAM during human liver development was investigated by immunohistochemistry. EpCAM cells were markedly highly expressed and localized mainly around the ductal plate area of 10-week liver,

confirming the presence of liver progenitor cells and corroborating with suggestion from previous studies done by Schelmz. At 14 weeks, more EpCAM positive cells were surrounded around the portal tracts and spread along the parenchyma of the liver. Interestingly, the expression of EpCAM expression is decreased during week 18, and upregulated in expression at 22 gestational weeks of the developing liver. (**Figure 2.2 and Figure 2.3**)

2.4.2 CD133 and CD44

The expression profiles of CD133 and CD44 marker for the liver specific stem cells were studied by immunohistochemistry. The expression of CD44 increased in week 10 and decreased at 14 and 18 weeks of the fetal liver. Then notably, there is second wave of CD44 and CD133 colabelled cells expression during the later phase in 22 weeks of fetal liver development. These data indicated the presence of liver specific stem cells during the early fetal liver development and a resurge in their regulation in later stages of liver development. (**Figure 2.4 and Figure 2.5**)

2.4.3 CD45: hematopoietic stem cell marker

Several varieties of stem cells, including mesenchymal stem cells and hematopoietic stem cells are found in the bone marrow. The CD45 marker is used to distinguish all hematopoietic stem cells for isolation and to grow as a homologous population (Shivtiel et al., 2008).

To understand hematopoietic expression during human fetal liver development, we co-labeled the slides with EpCAM and CD45 markers. The result distinguishes liver cells from hematopoietic stem cells. The cells, which

were stained for CD45, were not co-localized with EpCAM suggesting that these two markers were independently expressed. This expression pattern concludes that the hematopoietic stem cells are present in the developing liver. We observed that during fetal liver development, the number of CD45-positive cells displayed an interesting pattern – only peaking at 18 weeks, but remaining low at all other stages. These results coincide with the change in microenvironment niche of the liver that is conducive to hepatogenesis (**Figure 2.6**).

2.4.4 Albumin, AFP and KRT19

AFP is the most abundant plasma protein found in the human fetus. The expression of AFP has been reported to be decrease after birth where they begin its decrease prenatally (Jones et al., 2001). The differentiation of bi-potential hepatoblast into hepatocytes or Billiary epithelial cells (BEC) begins around E13 of mouse development. Initially hepatoblast express genes associated with both adult hepatocytes (Albumin) and BECs (cytokeratin-19), as well as in fetal liver genes such as α-fetoprotein (AFP).

The expression of AFP is interesting – we noticed a homogeneous expression at week 10 and an increase in the expression at week 14, which is believed to be the hepatoblast specification phase. AFP expression begins to decrease at week 18 and we notice a striking decrease in the expression at week 22. In contrast, the expression of albumin begins to steadily increase as the liver develops and we notice high expression of albumin cells at week 22 (**Figure 2.7**). The same expression pattern has been previously reported in rat liver during prenatal (17 to 21 days of gestation) and postnatal periods (1 to 5 weeks old neonates) (Poliard et al., 1986). During fetal stages (from week 14

up to week 18), about 50% of liver cells expressed albumin and AFP. From E18 up to birth, the albumin and AFP positive cells are expressed inversely; we observed an increase in albumin positive cells and a decrease in AFP positive cells, which disappears in adult liver (**Figure 2.7**).

KRT19 is a marker for billiary epithelial cells in the duct plate formation (Govaere et al., 2014). We notice the KRT19 cells are incorporated into the hepatic mesenchyme to form the biliary duct at week 10. Progressively, these cells begin to form as ductal plates at week 18. At 22 weeks, we see the formation of ductal plates surrounding the portal vein – an indication of maturation towards functional adult liver **(Figure 2.8)**.

Non parenchymal cells: How is nonparenchyma developed in fetal liver?

2.4.5 Vimentin and Smooth muscle actin

We analyzed the distribution of mesenchymal cells in fetal liver parenchyma. Here, the parenchymal cells were labeled with anti-Vimentin (mesenchymal cell marker) and anti-smooth muscle actins antibodies. The expression of Vimentin was distributed evenly throughout the liver development with no noticeable difference seen. However, we noticed that the expression of smooth muscle actins was increased at the vasculature of the blood vessel formation in liver. The formation of the vasculature initiates at around week 10 and forms an endothelial ring at around week 18. These events result in the formation of a blood vasculature at around week 22, leading to the maturation of fetal liver cells (**Figure 2.9 & Figure 2.10**).

2.5 Hierarchical clustering:

To define the genetically distributed phases in the liver development, total RNA was isolated from the human fetal liver samples, as described in the materials section. The RNA quality was analyzed with bioanalyser, upon which only those RNA samples that the passed the quality with integrity number (RIN) above 8 for each fetal liver samples were used for microarray analysis. An 8-bead chip Illumina microarray was performed and the raw data was analyzed with Partek analysis software. The Hierarchical clustering revealed that, adult liver to be in stark contrast with the expression pattern seen in all the fetal liver samples of the second trimester. Moreover, within the fetal liver samples, 10- and 22-week samples were closely related, as compared with 18- and 14-week samples (**Figure 2.11**). Further, the genotypic and phenotypic differences were analyzed to classify the differences between these phases with by qRT-PCR and immunohistochemistry.

2.6 Phases in human fetal liver development

Fetal livers RNA profiles were first subjected to customized qRT-PCR, which had genes, previously listed in liver development in animal and human models. In addition, the microarray analysis was performed systematically in fetal liver samples of various stages of development, in an effort to implicate novel genes in the role of fetal liver development in the second trimester. In both – qRT-PCR and microarray analyses, the hierarchical clustering revealed 4 distinct phases based on their expression pattern such as: stem cell regulation phase till week 10, hepatoblast proliferation phase at around week

14, hematopoietic phase at around week 18 and liver maturation phase at around week 22.

The key phenotypic and genotypic profile of each stage was analyzed using customized liver and stem cell gene array sets. The list of genes in these array sets is described in detail in materials and methods section. A few important genes that are expressed at different stages of fetal liver development are summarized in Table 1.

Table 3: Genes involved in different phases of liver development (Classified based on Known liver and stem cell Genes set)

Phases	Stem cell phase embryonic till week 10	Hepatoblast proliferation week 11 till week 14	Hematopoietic phase from week 15 till week 18	Liver maturation from week 19 till week 22 (MET phase)
Transcriptional factors	NANOG, NODAL, SOX2, OCT4 and HNF1a	SHH	XIST, TISX	NANOG, NODAL, SOX2, OCT4 ,HNF1a, HNF4a, SHH, GATA4
Growth factors	BMP4, BMP3, PDX1	BMP1, GDF2, WNT1	HGF, DLK1, OSM, BMP3	EGF, CTGF, BMP4, PDX1

Table 4: Overview of different stages in human liver development

10 week onwards	Stem cell and specification phase
14 week onwards	Specification

18 week – 22 weeks	Hematopoiesis and differentiation
22 weeks – adult	Maturation and proliferation

2.6.1 Phase 1 (10 week)

We observed that week 10 specimens were rich in embryonic stem cell transcriptional factor markers such as Homeobox Transcriptional factor (NANOG), NODAL Growth Differentiation factor, SRY Box2 (SOX2) and POU Class 5 Homeobox 1 (OCT4), which corroborates with the liver specification profile described in early studies **(Figure 2.12)**. At this stage we also noticed that the EpCAM positive cells were strongly expressed at the ductal plate area along with a higher number of mesenchymal stem cell marker-positive (CD44-positive) cells. These markers corroborate that the 10 weeks fetal liver is at the early stages of development and rich in stemness. We also observed the up regulation of Hepatic nuclear factor 1 (HNF1a) transcriptional factor that in literature described as a marker for hepatoblast specification (**Figure 2.13**).

2.6.2 Phase 2 (10 – 14 weeks)

Following the embryonic stem cell phase, the expressions of stem cell transcriptional factors were relatively reduced as compared to week 10. At this stage we do not observe any relative genetic changes compared to 10 weeks and 18 weeks samples. However, we notice three important markers upregulated, which are important for hepatoblast determination. At first, high number of AFP (Hepatoblast marker) positive cells was detected at this stage. In addition, a sudden surge in CD133 positive cell was observed along with

the increase in the level of Albumin expression was detected in the later stages.

2.6.3 Phase 3 (17 to 19 weeks)

It has been well documented that during fetal development, liver acts as a hematopoietic organ. In our study, we noticed the expression of CD45-positive cells (hematopoietic marker) at week 18.

2.6.3.1 Oncostatin M, TISX and XIST transcriptional factors

In addition, the expression of hematopoietic stem cell activator X-inactive specific transcript (XIST) was also upregulated. We considered this to be the hematopoietic phase. However, we also noticed the upregulation of TSIX (non-coding RNA gene that is antisense to the XIST RNA) gene. These changes at this phase, combined with the increased expression pattern of Hepatocyte Growth Factor (HGF), Oncostatin M (OSM) and delta-like 1 homolog (Drosophila) (DLK1) suggest that the hematopoietic stem cells are observed at week 18 of fetal liver development.

We noticed that the change was obvious with the expression changes with Oncostatin M, TISX and XIST. The expression of XIST transcriptional factor is believed to be transiently activated in hematopoietic stem cell differentiation. However, contrastingly, blocking the reactivation of hematopoietic stem cells resulted in an increase in the expression of XIST repressor factor TIST at week 18. This expression pattern of XIST and TIST at around week 18 in fetal liver suggests decrease homeostasis of hematopoiesis (**Figure 2.14**).

2.6.3.2 HGF: hepatocyte growth factor & Protein delta homolog 1 (Dlk1)

From the Array data, we observe the gene expression profiles of HGF and DLK1 gene were upregulated at around week 18 of fetal liver development. We have previously mentioned about the hematopoiesis during the 18 weeks period of the liver development, so by analyzing both the results, we believe that, when the hematopoietic stem cells secrete factors like OSM, HGF is activated to stimulate hepatoblast differentiation. These secreted factors induce the liver differentiation into liver maturation phase (**Figure 2.15**).

2.6.4 Phase 4 (19 – 24 weeks)

At the time that hematopoiesis phase is ceasing in liver, to our surprise we noted an upsurge in the expression of embryonic stem cell regulators factors such as OCT4, SOX2, NANOG, and the expression of KLF4 during the 22 weeks. This corresponded with an increase in the expression of CD44, an EpCAM progenitor marker. The expression of hepatic-specific transcriptional factors Hepatic nuclear Factor 4 (HNF4a), Hepatic nuclear Factor 3 (HNF3) and Prospero Homeobox 1 (PROX1) at this stage indicates that the liver undergoes maturation. This is surprising, as classically at this stage of liver development, hepatoblast differentiates into hepatocytes. We observed the downregualtion of AFP gene at 22 weeks compared to 10 weeks **(Figure 2.16)**. In addition, we also noticed the upregulation of EGF while other factors OSM and HGF decrease in expression (**Figure 2.17**).

At this stage, metabolic markers (CYP genes) begin to surge in expression during the week 22 of fetal liver development an indication of maturation (**Figure 2.18**).

2.7 Interpretation

This is the most systematic study yet, on the phenotypic and genotypic characterization of human fetal liver development. Temporal analysis of the developing fetal liver in second trimester, in this study, showed 4 distinct phases that are characterized based on the gene expression profile.

The expression pattern of EpCAM suggests that, there are distinct phases such as that of progenitor cell population surge at 10 weeks; that of hepatoblast proliferation from 10 to 14 weeks; hematopoietic phase at 15-18 weeks; and maturation phase at 22 weeks. This suggests that epithelial cell proliferation appears to take a backstage during the hematopoietic phase but undergo a second wave of progenitor cell proliferation with resurgence with termination of the hematopoietic phase.

Hematopoietic cells secrete OSM; and the expansion of hematopoietic cells results in the increase of the local OSM concentration, which consequently promotes hepatic development. In response to OSM, hepatocytes begin to acquire liver-specific functions at the expense of hematopoietic activity. XIST encodes noncoding transcripts that silence one of the 2 X chromosomes through X chromosome imprinting. XIST is considered to be active only at the early embryonic stages. The high-level expression of XIST supports the notion that XIST is reactivated during early hematopoiesis.

The termination of the hematopoietic phase can be explained by the following: 1) The loss of stromal cell phenotype which is believed to be the support cells for hematopoietic stem cells in liver (Kiel and Morrison, 2008). 2) The Unique expression of XIST and TIST gene at week 18. XIST gene is

shown to activate in hematopoietic precursor cells (Savarese et al., 2006) and TSIX gene is proven antagonist of XIST gene. This genetic imbalance in observed in week 18 only. 3) Although DLK and HGF gene are upregulated and show they are important in hematopoietic stem cell proliferation, it has been also showed that DLK inhibits formation of murine hematopoietic progenitors (Ohno et al., 2001). 4) It is shown that OSM supports both development of liver and hematopoiesis in the fetus but as hepatic cells mature, hepatic cells lose it ability to support hematopoiesis. Hence OSM is believed to support heptogenesis (Kamiya et al., 1999).

In conclusion, we have observed four different phases during the fetal liver development: the stem cell regulation phase at week 10; the hepatoblast proliferation phase at week 14; and the hematopoietic phase at week 18. At around week 22, the liver seemed to enter the maturation and expansion phase. Further, we also observed the presecnce of mesoendodermal cell population during week 22.

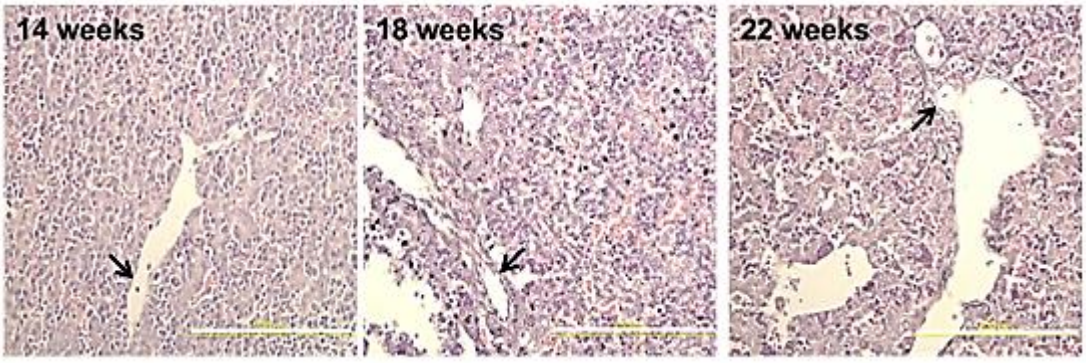


Figure 2.1 H&E – cellular architecture of fetal liver

The light microscopic images of H&E show the cellular architecture of liver. In our analysis of the fetal liver, the ductal plate (arrows) appeared at 14w and became more organized with portal mesenchyme (arrows) during 18w. By 22 weeks, portal triads (arrows) are formed and are surrounded by hepatocytes, which are arranged in single cell sheets known as hepatic plates, separated by sinusoid spaces that are connected to a network of blood vessels capillaries.

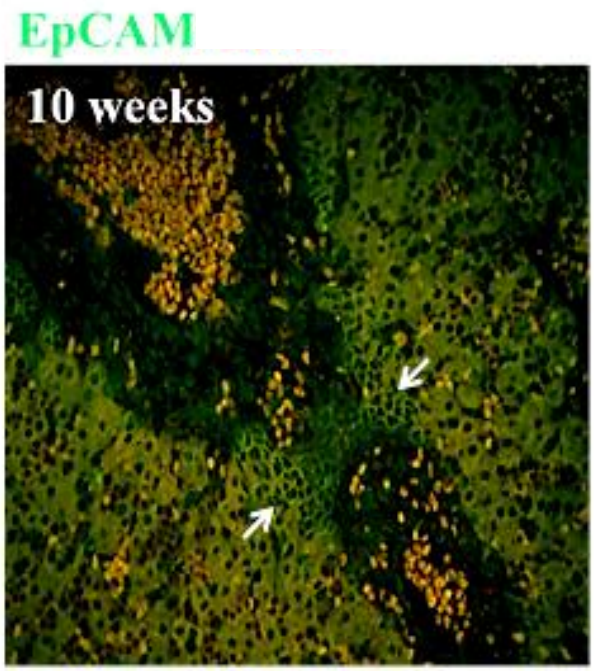


Figure 2.2 EpCAM expression at week 10

Immunofluorescence staining was performed on formalin fixed slides from developmental stage week 10. Cells (arrows) from week 10 are seen at ductal plate EpCAM (*green*) positive.

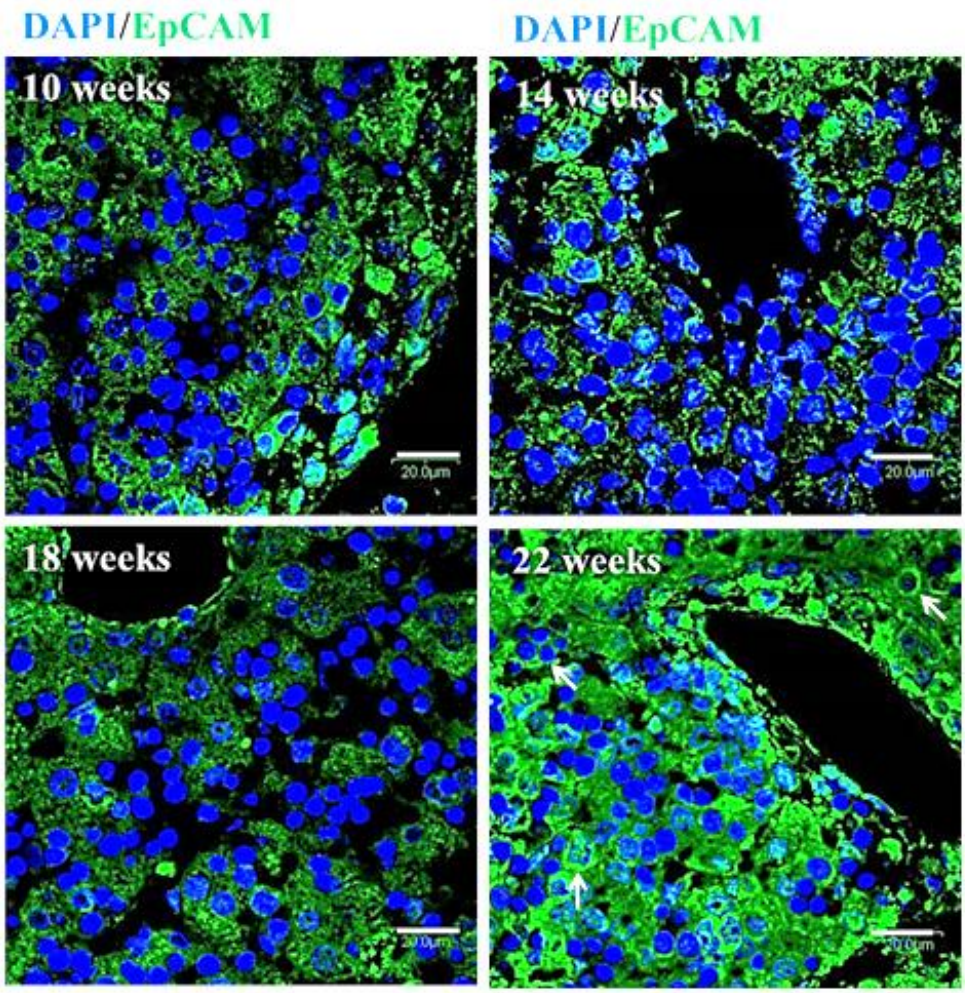


Figure 2.3 EpCAM expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. EpCAM cells were markedly highly expressed and localized mainly around the ductal plate area of 10-week liver confirming the presence of liver progenitor cells. From 14 weeks, more EpCAM positive cells were surrounded around the portal tracts and spread along the parenchyma of the liver. The expression of EpCAM expression is decreased during week 18, and upregulated in expression at 22 gestational weeks of developing liver. (Confocal Image). Scale bar 20 μm

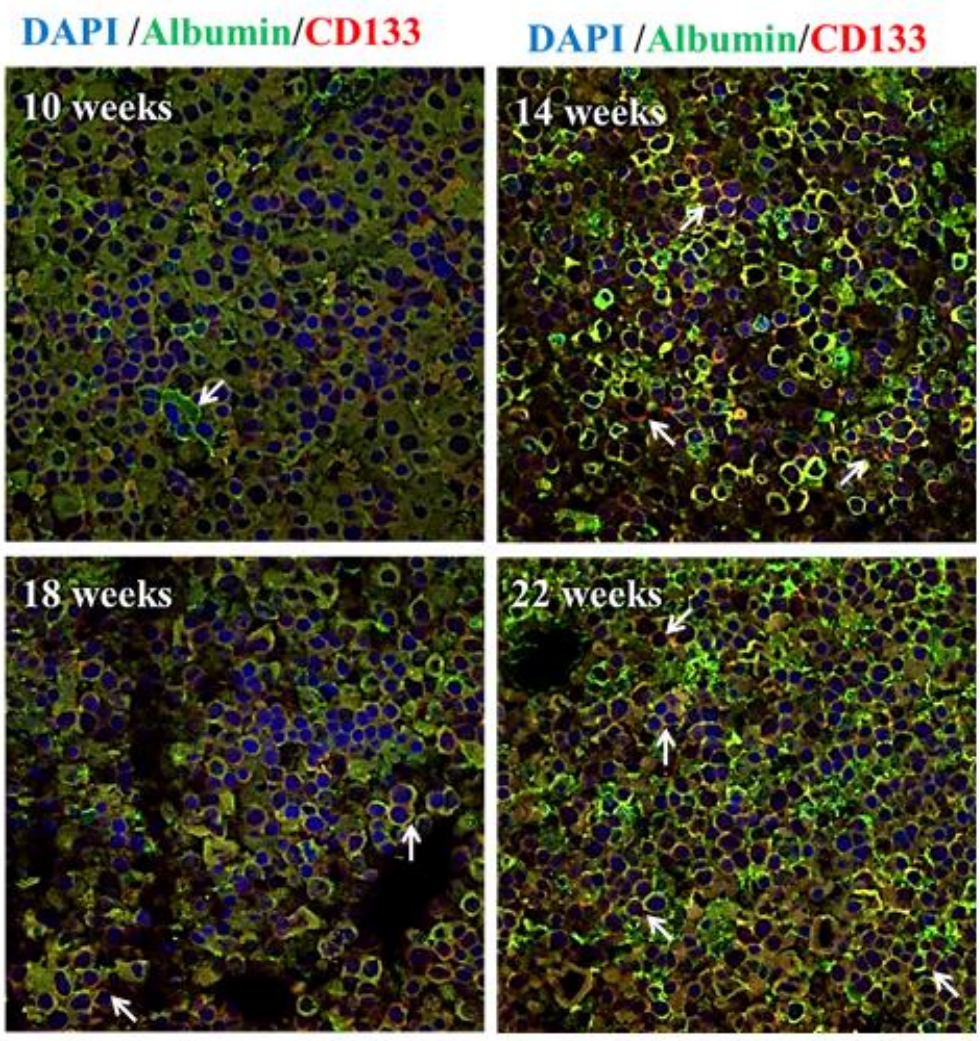


Figure 2.4 Alubumin and CD133 expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. CD133+ (green) albumin+ (red) colabelled cells (arrows) express strongly at week 14 indicating the presence of hepatic progenitor cells. In contrast at week 18 the percentage of CD133+ albumin+ cell populations is reduced, interestingly at week 22 these CD133+ cells are reduced noticeably.

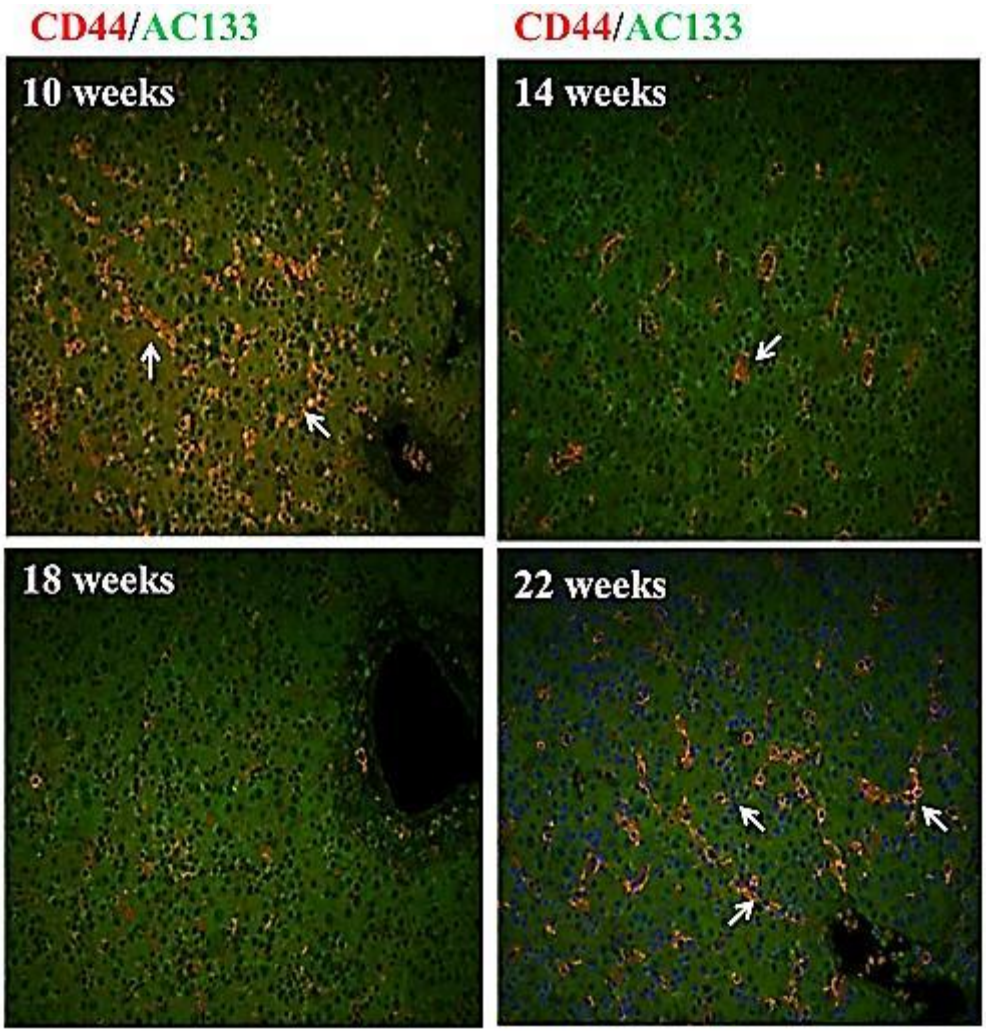


Figure 2.5 CD44 and AC133 expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. The arrows show the CD44 (red) cells strongly express in week 10. These cell number dwindle from week 14 to week 18. At week 22-second wave of CD44 cell populations are distributed into the parenchyma of liver.

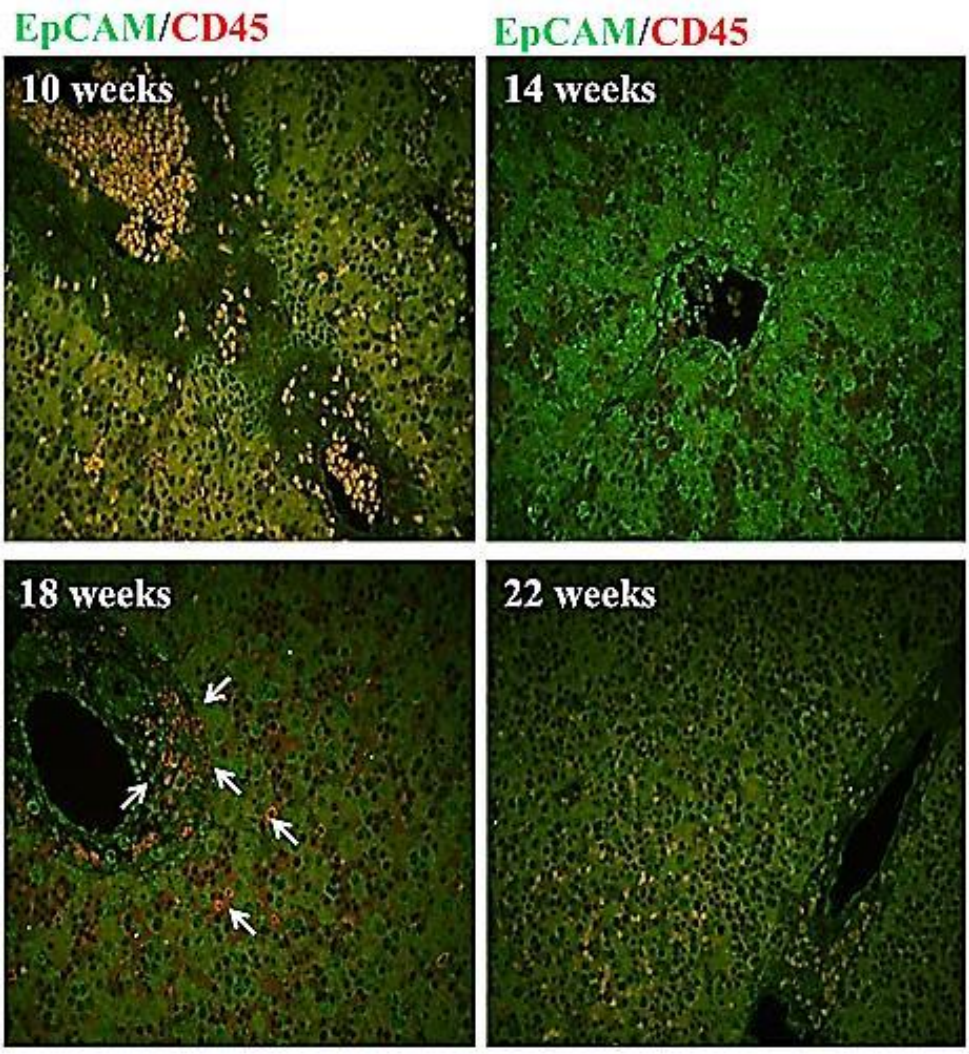


Figure 2.6 EpCAM and CD45 expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. The tissues were stained with EpCAM (green) and CD45 (red). Arrows show the CD45+ cells strongly express in week 18 and cell number dwindle from week 22 EpCAM (*green*) and CD45 (*red*) positive. It indicates that Hematopoietic stem cells highly express during week 18. Interestingly the CD45 cells did not co localizes with EPCAM marker.

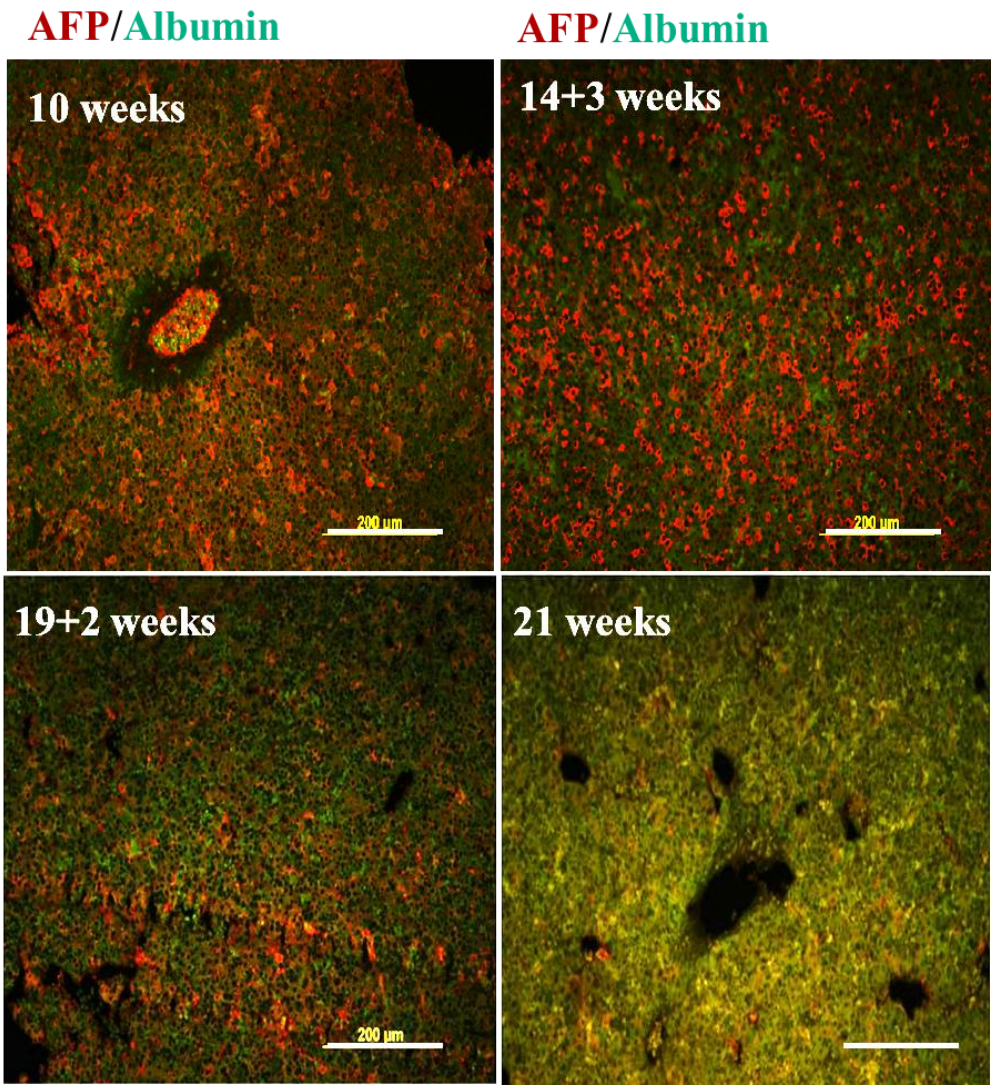


Figure 2.7 AFP and Albumin expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. The expression of AFP is interesting such as we notice an homogeneous expression at week 10 and increase in the expression at week 14 which is believed to be hepatoblast specification phase. AFP expression begins to decrease at week 18 and we notice a striking difference of decreased expression at week 22. In contrast the expression of Albumin expression begins to steadily increase as the liver develops and we notice high expression of albumin cells at week 22

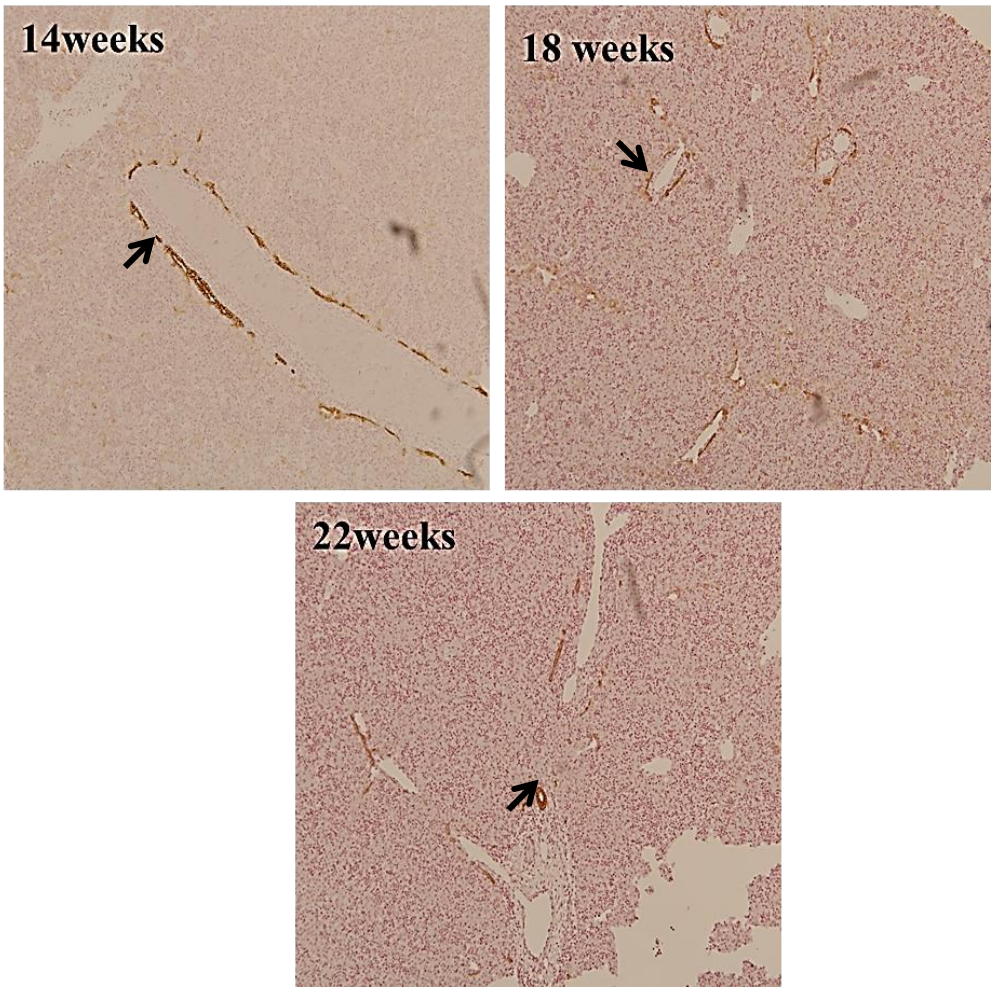


Figure 2.8 KRT**19 expression in fetal liver stages**

Immunohistochemistry reactions in formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. The KRT 19 is a marker for Billiary Epithelial Cells in the duct plate formation (arrows). We notice the KRT 19 cells are incorporated into the hepatic mesenchyme to form the biliary duct at week 10. Progressively these cells begin to form as ductal plates at week 18. At 22 weeks we see the formation of ductal plates (arrows) surrounding the portal vein, indication of maturation towards functional adult liver.

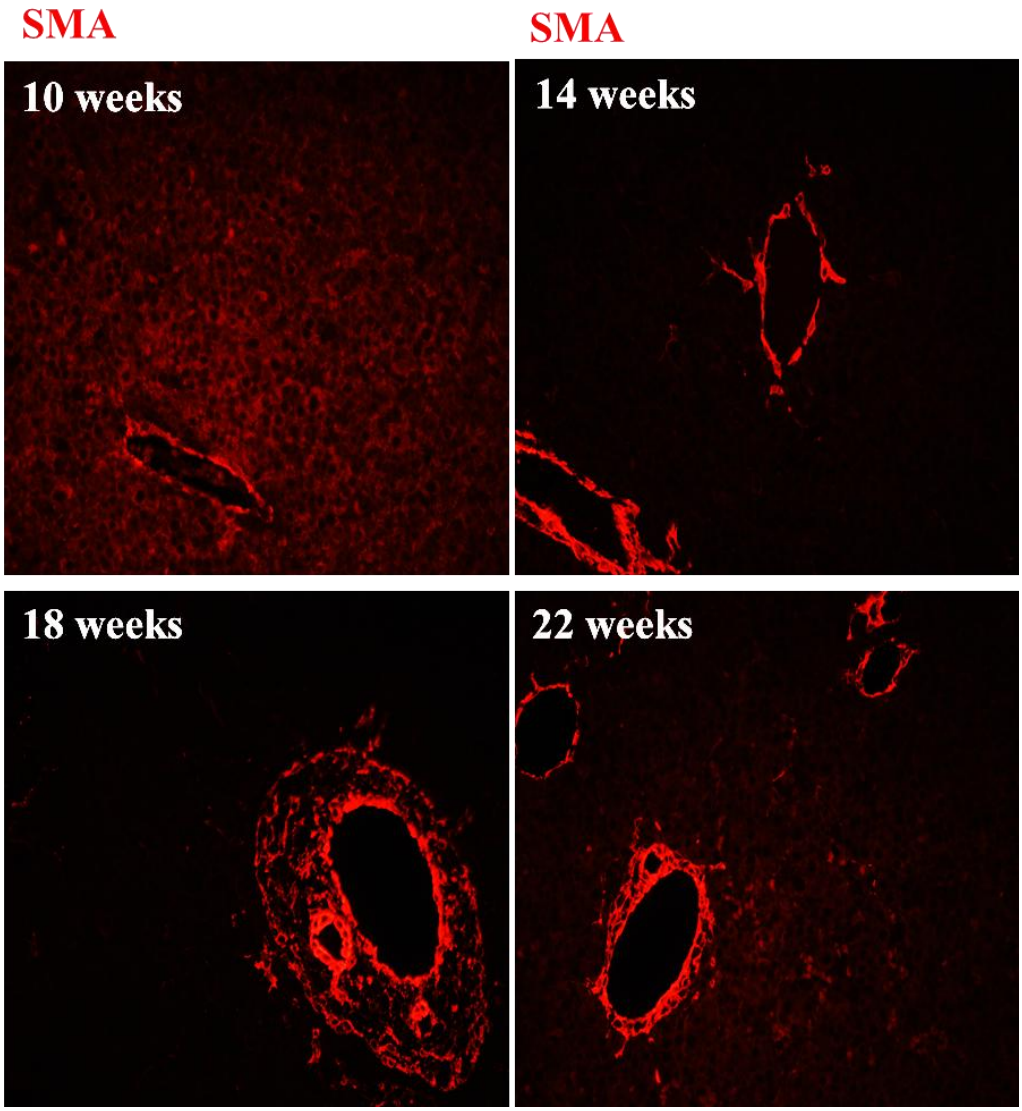


Figure 2.9 SMA expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. During fetal stages the smooth muscle vasculature formation begins at week 14 and matured cells are developed at week 22.

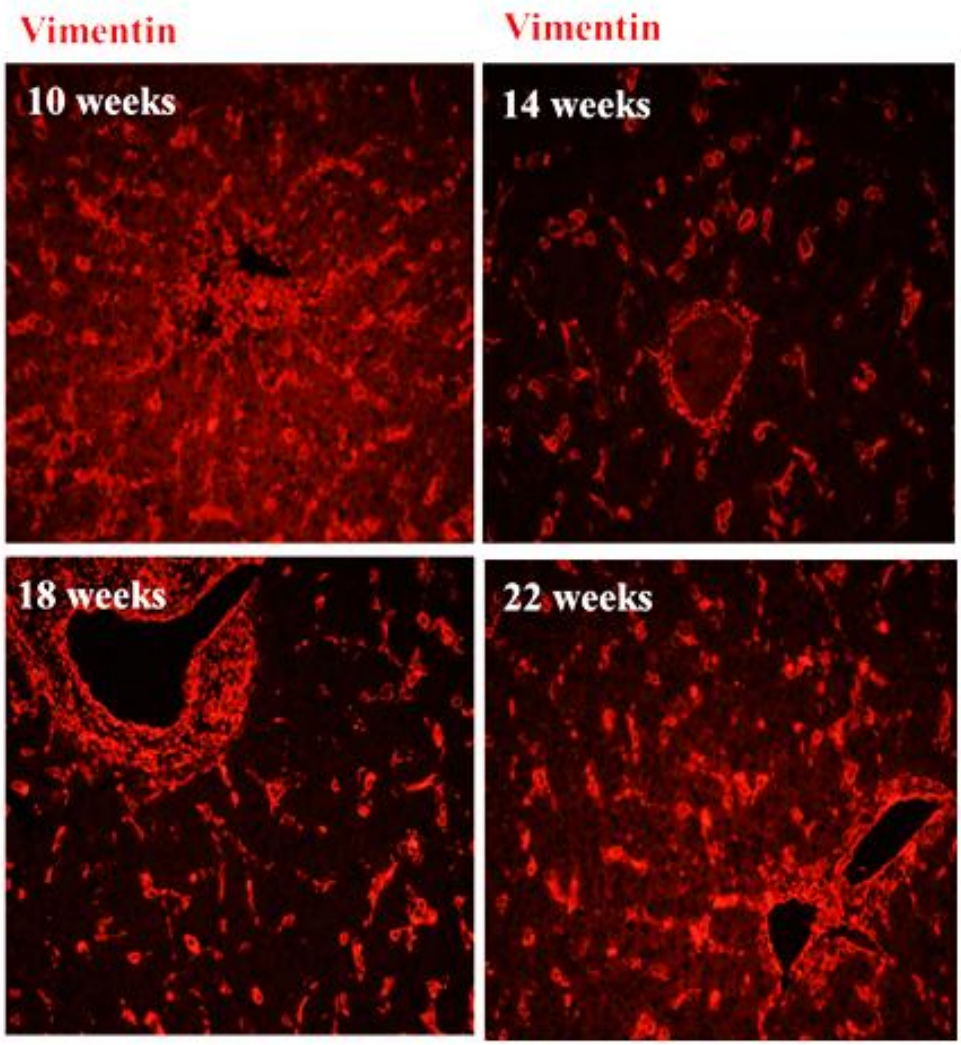


Figure 2.10 Vimentin expression in fetal liver stages

Immunofluorescence staining was performed on formalin fixed slides from developmental stages week 10, week 14, week 18 and week 22. During fetal stages the mesenchymal cells are evenly distributed throughout the liver development.

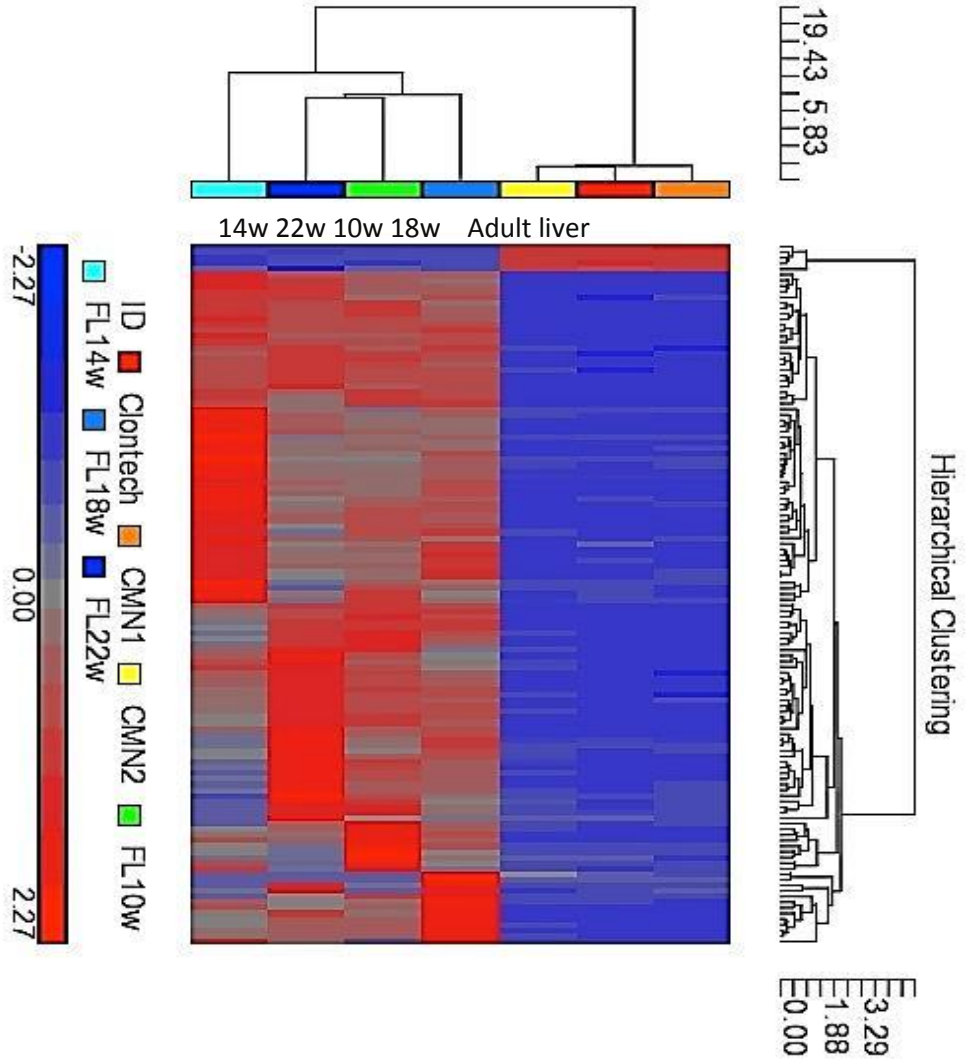


Figure 2.11 Hierarchical clustering analysis

Hierarchical clustering analysis was performed to group the human fetal liver samples. The figure represents the grouping of different stages of fetal liver. 10 weeks samples are represented as **green** color. 14 weeks samples are represented as **light green** color. Week 18 is represented as **light blue** color. Week 22 is represented as **blue** color. Adult liver is represented as **red** color. The fetal samples compared to Embryonic cells stage differentiation show distinct different from each other. Week 22 is similar to week 10 during development correlating with the second wave of gene expression compared to week 10.

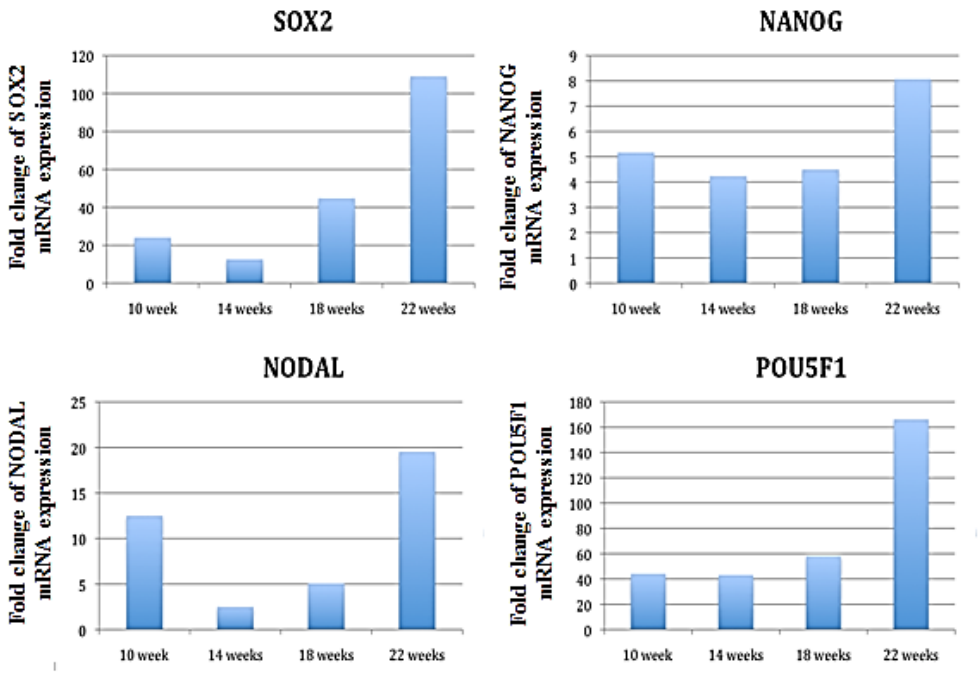


Figure 2.12 mRNA expression of embryonic stem cell factors

mRNA-expression levels of SOX2, NANOG, NODAL, OCT4 and KLHL1 embryonic stem cell factors at each developmental stages were measured by real-time-PCR array. The comparison was made with adult liver as control to show the level of expression. We noted a second wave of embryonic stem cells factors upregulated during 22 weeks of liver development. An similar trend is noted at week 10.

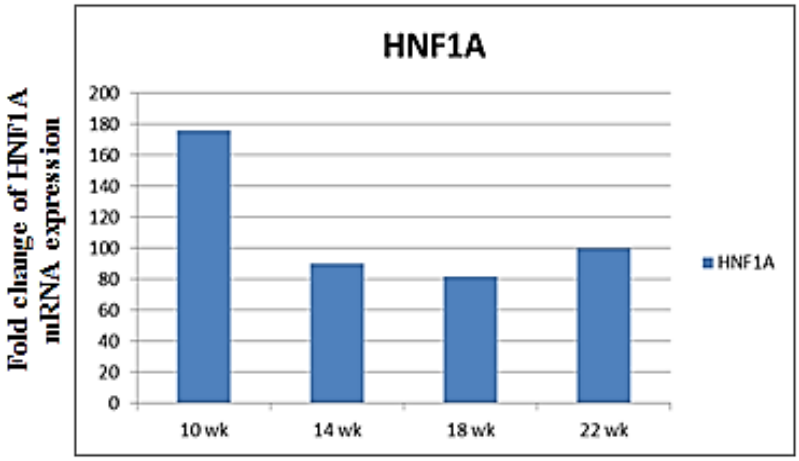


Figure 2.13 mRNA-expression levels of HNF1a

mRNA-expression levels of HNF1a at developmental stages were measured by real-time-PCR array. The comparison was made with adult liver as control to show the level of expression. We noted HNF1a gene was expressed at early stages 10 weeks of gestation indicating its importance in specification stage. The expression surge again at 22 weeks of liver development.

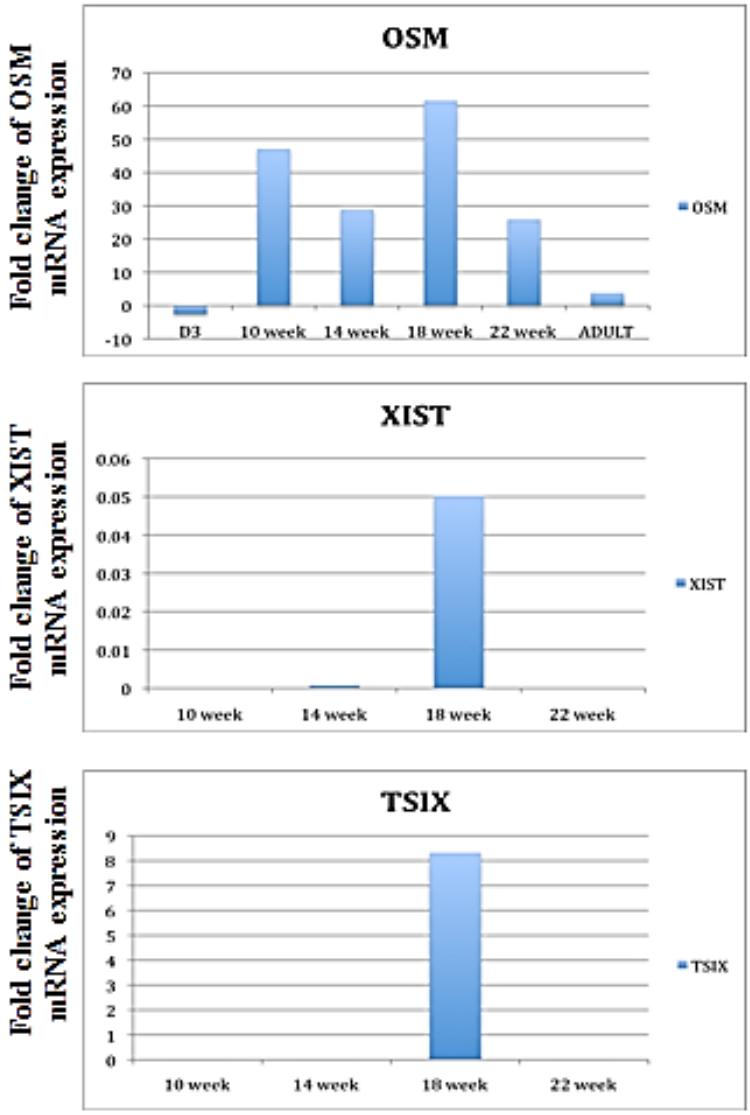


Figure 2.14 mRNA-expression levels of OSM, XIST and TISX

mRNA-expression levels of OSM, XIST and TISX at developmental stages were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. The expression was normalized with the endogenous control of GAPDH. OSM upregulated during week 18 and gradually reduce in expression as the liver matures. Interesting XIST (hematopoietic precursor cell activator) transcriptional factor was upregulated at week 18, interestingly we notices the upregulation of XIST repressor TISX transcriptional factor at week 18.

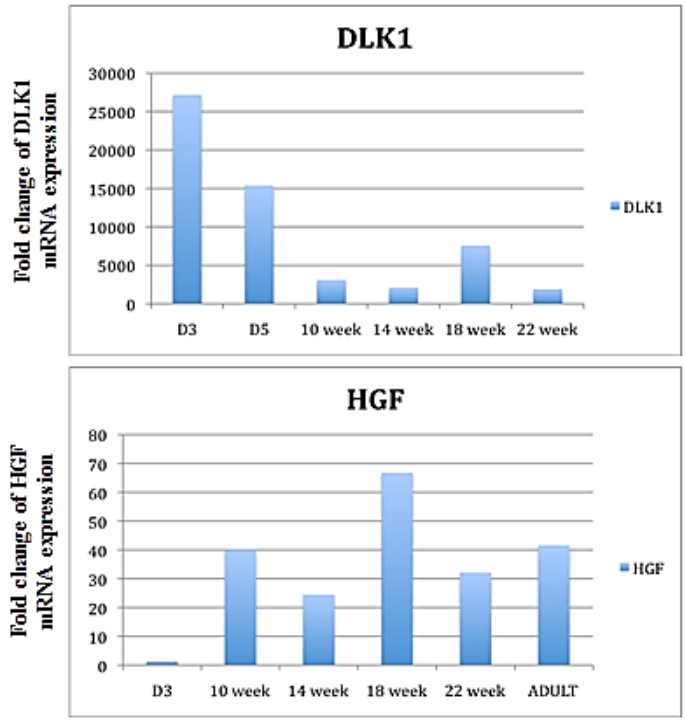


Figure 2.15 mRNA expression of DLK1 and HGF

mRNA-expression levels of DLK1 (Hepatoblast marker) and HGF (Hepatocyte differentiation factor) factors developmental stages were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. The expression was normalized with the endogenous control of GAPDH. Both DLK1 and HGF were upregulated during 18week of the human fetal liver development.

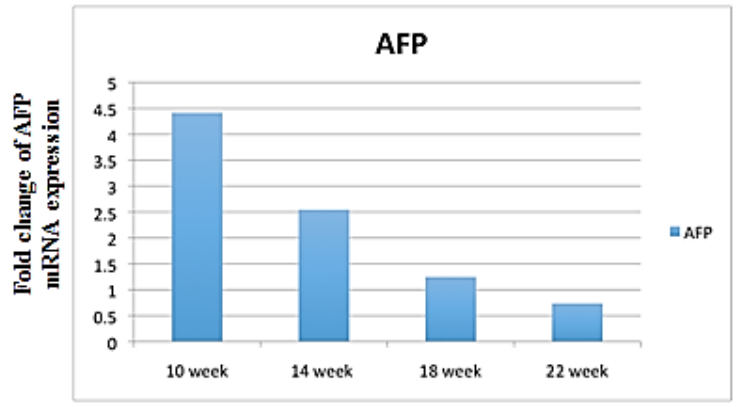


Figure 2.16 mRNA-expression levels of AFP

Regulation of AFP gradually decreases across the stages of fetal liver development indicating the maturation of liver.

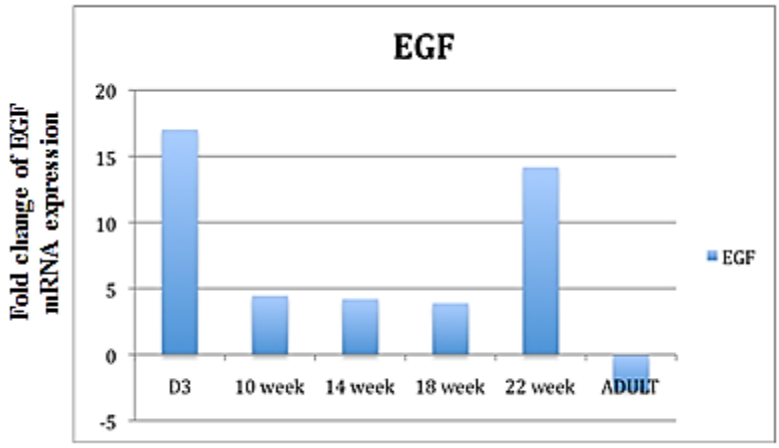


Figure 2.17 mRNA-expression levels of EGF

mRNA-expression levels of EGF factors developmental stages were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. The expression was normalized with the endogenous control of GAPDH. EGF expression is upregulated during 22 week of the human fetal liver development.

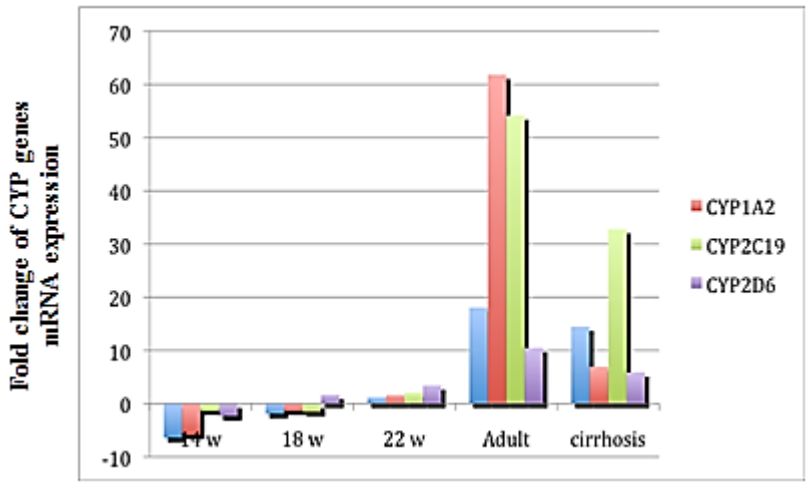


Figure 2.18 mRNA-expression levels of metabolic factors

mRNA-expression levels of metabolic factors CYP450 genes in developmental stages were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. The expression was normalized with the endogenous control of GAPDH. All these CYP gene markers were upregulated during the week 22 of the human fetal liver development. This validates that the liver begins to mature at after week 22.

Chapter 3

Transcriptional regulation of liver development

3.1 Background

In the previous chapter, we had classified and described different stages in human fetal liver development during second trimester. The study that we described in our previous chapter was based on the known liver and stem cell genes that were based on what has been reported in past research publications. To explore if novel genes and pathway may be involved in hepatocyte differentiation, we used full-genome arrays (next generation sequencing, microarray and PCR array) to identify transcriptional and micro-environmental factors (growth factors and extracellular factors) that may be differentially regulated during stages of the fetal liver development.

The recent advancement in high throughput genomics profiling give us an opportunity to identify key regulatory mechanisms such that it can define definite process for example stem cell or specification stage. These are the gene expression profiles, which include transcription factors and key hepatocytic genes. Gene expression profiling allows identification of genes that are differentially expressed between the fetal liver stages and allows the identification of unique markers. Understanding the upregulated or downregulated genes in fetal liver developmental phases will help us identify factors that may potentially play a role in the hepatocyte phenotype and those that regulate differentiation towards attainment of hepatic functions. The prospect of manipulating these transcription factors offers great potential for the transformation of progenitor cells to differentiated hepatic cells in *ex vivo* culture.

Current data available in the literature for gene expression profiles in liver development has been largely restricted to murine fetal liver or stem cell lines in models of liver disease. There is well-documented evidence and reasons to believe that the profile of human progenitor cells may be different from murine sources (Yu et al., 2010, Lin et al., 2014). A number of research groups have published the notable species differences in gene expression (Yu et al., 2010, Lin et al., 2014).

Studies, hitherto, lack detailed examination of various stages of liver development; but rather, deal with either detailed genetic or phenotypic examination at any one stage of liver development (Yu et al., 2001, Lee et al., 2012). In contrast, ours is a systematic study of gene expression and phenotypic characterisation in developing human fetal liver, using adult human liver as comparison.

Characterisation of genetic profiling of human fetal liver development and the maturation process as a high throughput screening of potential novel progenitor cell markers and key regulators of hepatocyte differentiation may add value to the current information about liver development. Knowledge gained from these studies will be critical as the potential for manipulation allows us to keep cells in their progenitor stage to achieve maximum expansion. Key genes that determine the liver phenotype will also form the basis for targets for subsequent attempts at reprogramming one's own somatic cell lineages to functional hepatocytes.

We hypothesize that comparing the genetic profile between different phases would provide clues to the regulatory genes that drive or at least are

associated with the different stages of liver development. Newly generated information on how new candidate genes fit into the genetic network will shed light on the complex regulation of liver development.

3.2 Materials and Methods

3.2.1 Source and isolation of human fetal liver cells

Human fetal livers were obtained from the KK women and children's hospital Singapore and at the National University Hospital of Singapore in accordance to the protocol approved by the institutional review board of both National university of Singapore and KK hospital (Table I). Written informed consent from the donor or the next of kin was obtained for use of all samples used within this study. Fetal liver was isolated and transported in DMEM medium.

Table 5:Number of samples used for the study

Gestational age	Number	Experiment
10 Weeks	3	NGS, Microarray, PCR array Immunofluorescence and FACS
14 weeks	3	NGS, Microarray, PCR array Immunofluorescence and FACS
17 week	1	NGS, Microarray, PCR array Immunofluorescence and FACS
18 week	1	NGS, Microarray, PCR array Immunofluorescence and FACS
19 week	1	NGS, Microarray, PCR array Immunofluorescence and FACS
21 weeks	1	NGS, Microarray, PCR array Immunofluorescence and FACS
22 week	1	NGS, Microarray, PCR array Immunofluorescence and FACS

23 week	1	NGS, Microarray, PCR array Immunofluorescence and FACS
Adult	5	NGS, Microarray, PCR array Immunofluorescence and FACS

3.2.2 RNA isolation

Total RNA was isolated using Trizol reagent (Invitrogen, Austin, TX Catalog number: 15596-026), 10 week, 14week, 18 week, 22 week and human adult liver. Isolated RNA was subjected to bioanalyser to check the integrity and used which was more than 8 in all our experiments.

Materials and instrument:

Trizol reagent (Invitrogen, Austin, TX Catalog number: 15596-026) 70% ethanol β-mercaptoethanol Spectrophotometer (Nanodrop; Model No. ND1000, Thermo Scientific, MA, USA)

Procedure:

Homogenization: Clean Polytron homogenizer with RNzap, then wash with EtOH, dH2O and finally with 1 ml of Trizol reagent (every time run Polytron at full speed for about a minute). Add 500ul Trizol into a 5ml tube. Transfer freshly isolated liver tisues to an eppendorf tube, Add with Trizol (500ul), pool together to bring the volume to 1ml total.

Phase separation: Shake the homogenate for 10 minutes at RT to permit the complete dissociation of nucleoprotein complexes. Supplement the homogenate with 0.2ml of chloroform per 1 ml of Trizol, cover the samples tightly and shake vigorously (vortex) for 15 seconds. Incubate the mixture at RT for at least 10 minutes (up to 30 min). Centrifuge at 14, 000g for 15

minutes at 4^{o}C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 60% of the volume of Trizol used for homogenization**.** Add isopropanol and the high salt solution (0.8 M Sodium Citrate, 1.2 M NaCl), each at one half volume of the aqueous phase. Keep samples at –20 oC O/N (or at least 1 h). Centrifuge at 14, 000g for 30 minutes at 4 oC.

RNA wash and Solubilization: Discard the supernatant. Wash 2x with 1.0 ml of 75% ethanol per 1ml TRIzol used for the initial homogenization. Vortex, then spin down at 7, 500 rpm/5 minutes at RT. Remove ETOH. Let RNA pellet briefly dry. Dissolve in 20-30ul diH2O Depending on the amounts, the resultant total RNA may be subjected to further clean-up with Qiagen RNEasy columns. However, it is not recommended if we use get less than 10 ug of total RNA (expect 20-40 % loss from the clean-up). Quality controls using Agilent Bioanalyzer.

3.2.3 Qualitative Real Time - PCR Array

We have used a set of known liver genes that were described in previous studies. We customised the PCR array with 364 genes in 4 different plates. These plates were classified as 1. liver and stem cell genes, 2. Growth factors and 3. Extracellular matrix. These customised plates were manufactured by sabiosciences.

Principle

Quantitative real time RT-PCR (qRTPCR) is used to amplify a certain region

of cDNA using forward and reverse oligonucleotides (primers) specific to that region. The amplified double-stranded DNA is quantified by measuring the amount of fluorescence dye, such as SYBR green, that intercalates with the DNA.

Firstly, the two strands of DNA are separated to form two template strands by heating at a high temperature for a brief period of time. This is followed by amplification process during which the primers bind to the template strands and the enzyme, taq DNA polymerase extends the specific region of template DNA. The primers are designed such that they are complementary only to one specific region of the DNA which represents a particular gene of interest. Each amplification cycle has alternating temperature to allow the sequential process of primer binding, extension and denaturation. After several cycles of amplification, adequate copies of cDNA are made.

During the amplification process, SYBR green fluorescence dye intercalates with double stranded DNA and the qRTPCR instrument quantifies the fluorescence intensity of the intercalated dye and provides a numerical value called the cycle threshold (Ct) value (Bustin 2000). The $2^{-[\Delta\Delta Ct]}$ method is used to calculate the relative expression of a particular gene in experimental groups when compared with control groups (Livak and Schmittgen 2001).

25mg of RNA were reverse transcribed (SABiosciences first strand kit Catolgue No - 330421), and PCR array was performed with 364 genes that were divided into four different 96 well plates. This was used to determine expression of several genes using specific primer pairs.

3.2.4 Illumina microarray analysis

Once we have classified the known set of liver genes, we then probed for the high throughput gene analysis.

3.2.4.1 Principle of microarray experiment

A microarray experiment is performed to identify gene expression changes on a high-throughput basis. This procedure involves the basic principle of nucleic acid hybridization. A microarray chip is made up of thousands of oligonucleotides attached onto a solid surface. RNA is extracted from the sample to be profiled and converted to cDNA by reverse transcription. The cDNA is converted to cRNA by in vitro transcription with fluorescence or biotin tags. The biotin-labeled cRNA is spotted on the microarray chip for hybridization for a certain period of time and the non-hybridized cRNA is washed away. Finally, the microarray chip is scanned under laser light and the images obtained are analyzed (Heller 2002; Murphy 2002).

3.2.4.2 Sample amplification and hybridization

RNA (500ng) was amplified using the Illumina Total Prep RNA Amplification Kit, according to manufacturer's instructions. cRNA was hybridized to Illumina Human Sentrix-6 bead chips Ver.2, according to standard Illumina protocols (http://www.illumina.com).

3.2.4.3 Raw data curation and normalization of Illumina microarray

Chips scans were processed using Illumina BeadScan and BeadStudio software packages and summarized data was generated in BeadStudio (version 3.1). The summarized data from BeadStudio was imported into R/BioConductor using the read Bead function from the Bead Explorer

package Background adjustment and quintile normalization was performed using algorithms within the Illumine package (function: bg. adjust and normalize. quintiles). The normalized data was exported out off R/BioConductor with write. BeadData function.

3.2.4.4 Normalization and statistical analysis of Illumina microarray data

All microarray experiments were conducted in biological triplicate. A gene was considered detected if the average detection score (p-value) of the three replicates was less than 0.05. Quintile normalization and statistic calculations were carried out using the Partek software.

3.2.5 Next generation sequencing data

Our analysis from PCR array and Microarray data showed different phases in liver development (Discussed later in the chapter). We need an advanced tool to analyze the important genetic differences at each stage. Although microarray provided us a large amount of data the limitation of using the denominator of genes greater that 2 fold restrict us to existing genomic sequencing information. RNA-seq experiments on the other hand work well for investigating both known transcripts and exploring new ones. Therefore, RNA-seq is ideal for discovery-based experiments. And while microarrays are limited to the reference information available during production, RNA-seq experiments may be updated as new sequence information is obtained.

Second, RNA-seq delivers low background signal. This is because DNA sequences can be unambiguously mapped to unique regions of the genome. As a result, noise in the experiment is easily eliminated during analysis. Hybridization issues seen with microarrays, such as cross-hybridization or

non-ideal hybridization kinetics, are also eliminated in RNA-seq experiments. This offers another signal-to-noise advantage.

Finally, RNA-seq has the ability to quantify a large dynamic range of expression levels, with absolute rather than relative values. Even with organisms lacking a reference genome, de novo transcriptome assembly and differential expression analysis can be performed. There is no upper limit for this quantification, and at Cofactor, we can help you to determine the number of reads you need to find the information you're looking for with high levels of reproducibility between both technical and biological replicates.

For the next generation RNA sequencing data, we isolated RNA from the tissue samples determined the RIN number and passed on to our collaborator in Genome Institute of Singapore for the sequencing.

Results:

3.3 qRT-PCR array:

Gene expression was measured across the fetal liver samples, in second trimester, with 10 weeks liver sample as control. The genes that are 2-fold upregulated were considered significant. Based on our observation, the data is presented in three different sections (transcriptional factors, Growth factors and Extracellular factors).

This PCR array study will only validate the genes, associated with previous liver development studies. On the other hand, we could determine the temporal expression of these specific genes in each of the described phases of human fetal liver development.

3.3.1 Transcriptional factors at different phases of fetal liver from qRT-PCR array data

The transcriptional factors studied in our customized PCR array data are genes from previous studies on murine liver development. Liver specification genes such as HNF1A, HNF1B PROM1 were expectedly highest at 10 weeks when the liver bud is formed and is downregulated at 14 week and 18 week compared to week 10. Interestingly, several key hepatocyte differentiation genes including HNF4α, PROX1 and HNF1B showed a bipolar upregulation at week 10 and then gets expressed later at weeks 22 and adult liver, corresponding to our observed 2 stage epithelial expansion in the fetal liver. Both HNF4α and PROX1 have been shown to be master regulators of novel regulator of cell differentiation and morphogenesis during hepatogenesis. These are supported by reported work in knock mice, which exhibited dramatically reduced expression of multiple hepatocyte genes and hence, very defective hepatocyte morphogenesis (Seth et al., 2014). Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment. We also observed that C/EBPα and C/EBPb – early liver development markers, appear to be down regulated during fetal liver development and upregulated in adult liver compared to the week 10 as control, again supporting data that the fetal liver is highly immature compared to adult liver and the importance of these genes in hepatocyte differentiation **(Figure 3.1A and Figure 3.2A)** (Soriano et al., 1995)**.**

Along the same vein, we noted the gradual increase in GATA 4-6 gene expression during liver development compared to stage week 10, underlying

their key roles in liver development. We also looked at nuclear signals that have been reported to be highly conserved in organogenesis in multiple species. Comparing the various SMAD genes, we noticed that SMAD 5 was significantly upregulated at week 10 and significantly downregulated throughout the liver development at each of the other time points. In contrast, SMAD 3 had an upward trend of gradually increasing in gene expression from week 14 till adult liver compared to week 10. The roles of these SMADs have not been fully elucidated in hepatogenesis and offer a potential regulatory pathway of upstream TGFbeta signaling in liver progenitor cell specification **(Figure 3.1B and Figure 3.2B).**

3.3.2 Growth factors from qRT-PCR array data

Significantly BMP 2 expressions were highly upregulated at week 14 compared to week 10. It has been previously reported that BMP2 determines hepatic vs. pancreatic fate (Won Suk Chung et al., 2008). We noticed an upregulation of BMP1 gene at adult liver compared to week 10 **(Figure 3.3A)**.

We noticed that FGF genes were comparatively downregulated in fetal liver stages compared to week 10 with the exception of FGF1. FGF1 was highly upregulated at week 14 compared to week 10 and had a downward trend in gene expression during liver development, suggesting a potential association with hepatoblast differentiation and amplification. Interestingly we noticed the upregulation of FGF4 in adult liver compared to week 10. It was shown in previous studies that FGF4 is a key regulator of hepatocyte precursor differentiation (Sekhon et al., 2004) **(Figure 3.3B)**

3.3.3 Extra Cellular Matrix

The specific ECM factors that were differentially regulated were identified as Fibronectin, Hyaluronic acid and collagen12A1, which were upregulated at 14 weeks compared to week 10, suggesting potential for exploitation in expanding hepatoblast *in vitro*. Factors like Lamanin 3 were upregulated at 22 weeks compared to 10 week raising its possible role in shaping the microenvironment niche for proliferation of progenitors at 22 weeks **(Figure 3.4).**

3.4 Next generation sequencing analysis:

Next generation sequencing was performed to identify the uniquely expressed genes at each stages of fetal liver development.

3.4.1 Genetic categorization of fetal liver and adult liver:

We analyzed the data with all stages of the fetal liver as one group versus adult liver. We observed 518 genes only enriched for fetal liver and 412 specific for adult liver. The top 20 genes that were exclusively expressed in fetal and adult liver are shown in **(Figure 3.5).** We proceeded to validate the exclusive expression by using RT PCR of AFP gene in fetal liver and CYP3A4 in adult liver.

3.4.2 Expression of transcriptional factors at each stage of fetal liver development

NGS analysis was done and the top transcriptional factors expressed at each stage of fetal liver development were identified. From the analysis, we observed upregulation of embryonic stage transcriptional factors at week 10 such as GATA2, HNF4a and SMARCA2 genes. At week 18, genes that were upregulated included GLI2 and FOXC1, critical regulators of hematopoietic stem cells; HOXD9, E2F1 which have been reported to be involved in liver

development but remains elusive; and LEF1 involved in T cell development (Omatsu et al., 2014; Li Wang et al., 2014; Yu et al., 2012). In week 22 we have noticed the upregulation of FOXL1 a hepatic progenitor specific marker (Shin et al., 2011). PPARG and STAT3 genes are involved in hepatocyte differentiation, along with GATA2, FOS and CREB genes (Ito et al., 2000). In corroboration with our PCR array data, the NGS showed upregulation of CEBPa/b, HNF4a, GATA1/2/3 and SOX10 expression at adult liver validating the importat of these genes in liver maturation **(Figure 3.6)**.

3.4.3 GO analysis

Next generation analysis was performed and the genes with p-value less than 0.05 was used for this analysis. The top 500 genes were identified at each stage; these 500 genes were further analyzed and the unique genes for each stage were identified and were performed for GO analysis with David GO database. The significant signaling pathways that were regulated during each stage were analyzed. To categorize the pathways altered during liver development, KEGG and GO analysis from DAVID open database was used. The genes at each time point were separated into those that were up or down regulated relative to the adult liver, and were analyzed separately.

The functional groups from week 10 till week 18 involved in **cell cycle, mRNA processing, ribosome activity**, cellular homeostasis and MAPKKK regulation. In week 22 Pathways were related to mesoderm development, **regulation of lymphocyte activation**, metabolic process, embryonic development and Regulation of T cell activation **(Figure 3.7)**.

The KEGG pathway analysis implicated calcium signaling pathway in week 10; cell communication, **gap junction** and long-term potentiation in week 14; and hedgehog-signaling pathway in week 18. Interestingly, in week 22 we notice **hematopoietic cell lineage, hedgehog signaling pathway** and renin angiotensin system **(Figure 3.8)**.

3.4.4 Gene atlas

Gene atlas analysis shows the fetal liver express early erythroid, endothelial and fetal liver gene expression, whereas in adult liver, the genes expressed are specific to liver only.

3.5 Liver specific markers

Many studies have used classical hepatic markers such as albumin and SERPINA as surrogate markers for matured hepatocytes. However our data suggest that mRNA of both these genes are already upregulated in liver progenitors that have yet to fully mature. Along this line, we analyzed for the genes that were specific for mature hepatocytes only in the adult liver. RNA-seq profiles from 16 human tissues from public databases **(Figure 3.9)** were analyzed for Liver specific genes. The final gene list showed **160** highly expressed liver-specific genes and identified among these; ALB (albumin) and SERPINA1 (Alpha antitrypsin) **(Figure 3.10)** are listed as examples. These 160 liver specific genes that are enriched mainly in complement immune system, drug metabolism, CYP450 and serine proteinase inhibitors. These genes are highly expressed in adult liver tissues but they may be also expressed in fetal immature liver cells and remains highly expressed in mature adult liver cells. From our next generation sequencing, we obtained

top 5% highly expressed genes that are identified in each of these samples and mapped them to the 160 liver-specific genes from the public database **(Figure 3.11)**. Our data showed ALB is highly expressed in as early as 10-week old fetal liver; thus, we propose that ALB mRNA as a marker of mature hepatocytes be interpreted with caution. We have identified 12 adult liver specific genes are highly expressed only in the adult liver samples, namely, ORM1, ORM2, APCS, HPX and APOC2 **(Figure 3.12)**, which can be used as markers for mature adult hepatocytes.

3.6 Summary

1. G0 and KEGG pathway analyses further strengthen our classification of mid-trimester liver development into distinct stages and enhance our understanding of development.

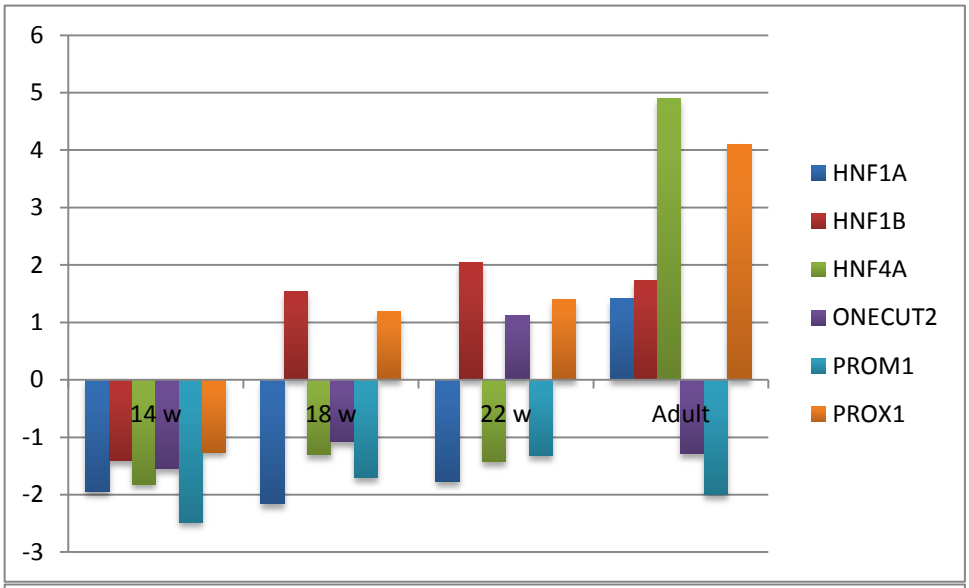
2. Key genes that we identified at each time point could serve as potential targets for *ex vivo* manipulation.

3.7 Interpretation

Our finding shows that data correlates with the previous findings that have describes the liver development in other animals. The transcriptional network remains the same in both the human and animal model of fetal liver development. However the interesting facts that we highlight in our results are the time points that they regulate. HNF4, HNF1 and PROX1, the crucial transcriptional factors for the hepatic differentiation, are expressed at 22 weeks of fetal stage. This is coordinated with other important transcriptional factors such as C/EBPa that was earlier believed to suppress hepatoblast proliferation high upregulated during maturation stage. GATA4 gene, that

which is expressed in early embryonic stages, that which determines the hepatic bud formation, is also highly expressed at around week 22. The stem cell transcriptional factors such as SOX2, OCT4, NODAL and NANOG are upregulated at week 22. These data show that the liver undergoes tremendous change at the late stages of development. This could be the key phase that will help understand the production of matured functional hepatocytes.

A



B

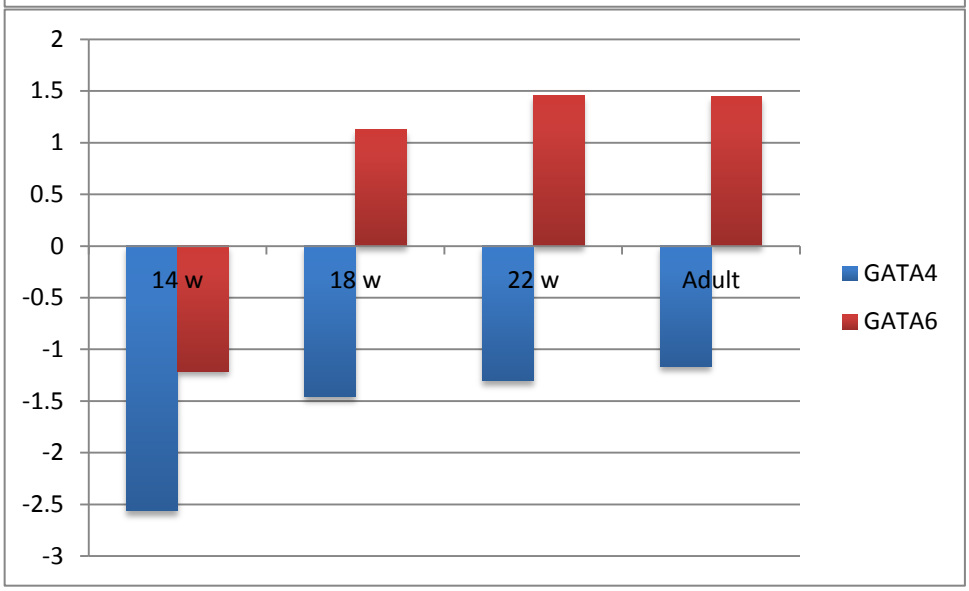
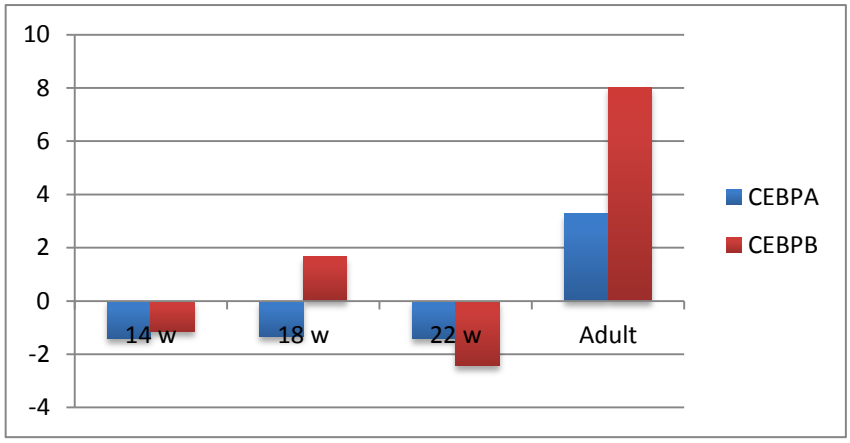


Figure 3.1 mRNA-expression levels of liver specific transcriptional factors

mRNA-expression levels of transcriptional factors in developmental stages were measured by real-time-PCR experiment. The expression was normalized with the endogenous control of GAPDH. **A.** Liver specific transcriptional markers such as HNF1A, HNF1B PROM1 were downregulated at 14 week and 18 week compared to week 10. HNF4α, PROX1 and HNF1B showed striking difference, which expressed at week 10 and then expresses at later weeks 22 and adult liver only. **B.** We also observed that gradual increase in GATA 4-6 gene expression during liver development compared to stage week 10.

A



B

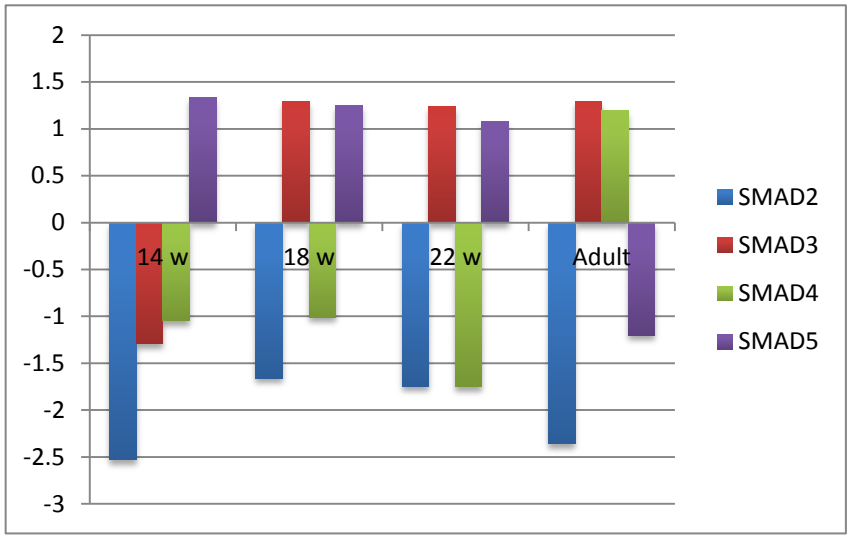
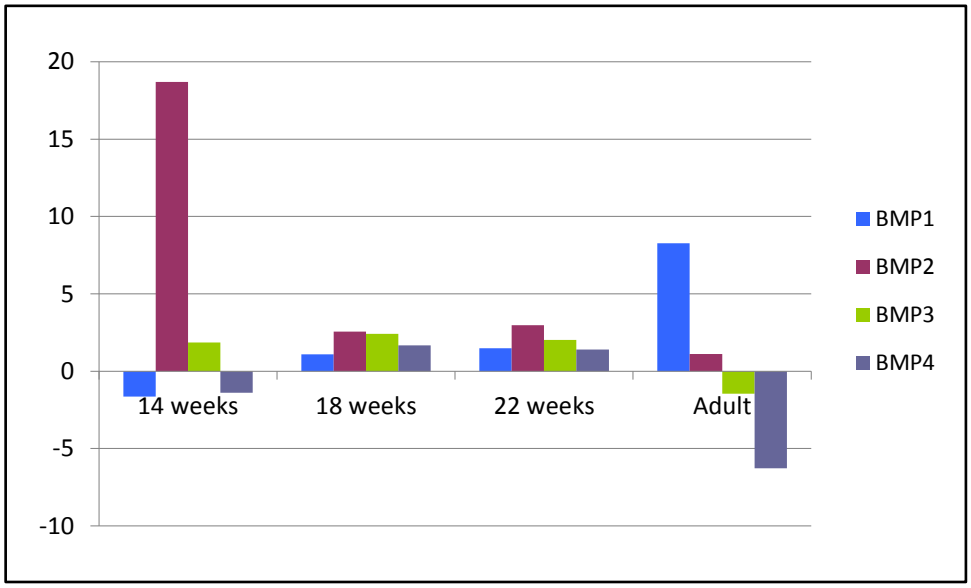


Figure 3.2 mRNA-expression levels of liver specific transcriptional factors

mRNA-expression levels of transcriptional factors in developmental stages were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. The expression was normalized with the endogenous control of GAPDH. **A.** C/EBPα and CEBPb early liver development markers (Soriano et al., 1995) appear to be down regulated during fetal liver development and upregulated in adult liver compared to the week 10 as control. **B.** SMAD 5 upregulated at week 10 and significantly downregulated throughout the liver development at each time points compared to week 10. In Contrast SMAD 3 had an upward trend of gradually increasing in gene expression from week 14 till adult liver compared to week 10.

A



B

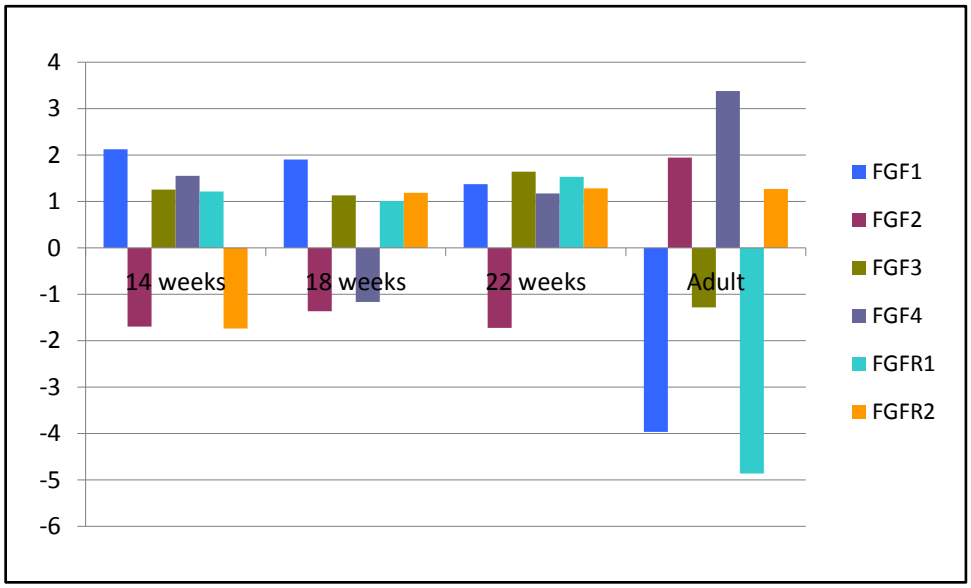


Figure 3.3 mRNA-expression levels of growth factor signaling

mRNA-expression levels of growth factor signaling in developmental stages 14 weeks, 18 weeks, 22 weeks and Adult liver were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. **A.** We observed BMP gene expression at each stage of liver development. Significantly BMP 2 expressions were highly upregulated at week 14 compared to week 10. Interestingly we noticed an upregulation of BMP1 gene at adult liver compared to week 10 **B.** We noticed that FGF genes were comparatively downregulated in fetal liver stages compared to week 10. FGF1 was highly upregulated at week 14 compared to week 10 and had a downward trend in gene expression during liver development. Interestingly we noticed the upregulation of FGF4 in adult liver compared to week 10.

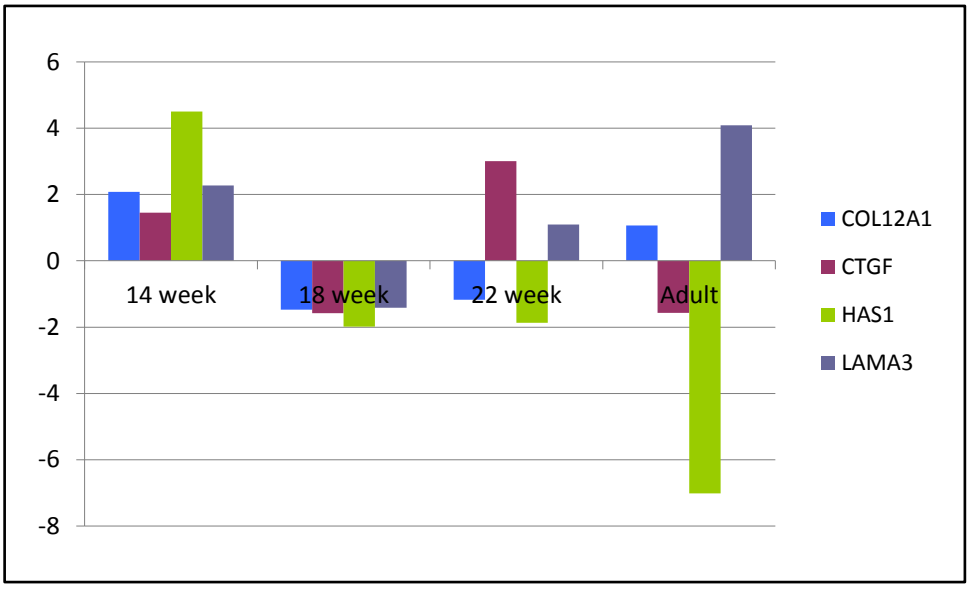


Figure 3.4 mRNA-expression levels of extracellular matrix signaling

mRNA-expression levels of extracellular matrix signaling in developmental stages 14 weeks, 18 weeks, 22 weeks and Adult liver were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. Fibronectin, Hyaluronic acid and collagen12A1, which were upregulated at 14 weeks compared to week 10. Factors like Lamanin 3 were upregulated during 22 weeks compared to 10 week as control.

Fetal Liver enriched	Adult Liver enriched
21	**21**
IGF2	LOC100132529
HBA2	HP
HBG2	ORM1
HBA1	HEPN1
H19	ORM2
AFP	TARDBP
TMEM200B	CYP2E1
PRC1-AS1	LOC100422737
CCNDBP1	IGF2-AS
CERKL	NDUFA6-AS1
INS-IGF2	RHEB
CDR1	SERPINA3
AHSP	LRRC8A
MT1G	UTS2B
PEG3-AS1	MRTO4
RPL17	C1R
HMGB2	CES1
RPL5	ACOT6
RPL14	LOC100128531
ENOSF1	KLF9
PTOV1-AS1	ADH1B

Figure 3.5 Uniquely expressed genes in fetal liver vs. adult liver

Uniquely expressed genes in fetal liver vs. adult liver based on relative expression in Next Generation RNA sequencing analysis. AFP gene is expressed only in fetal liver and SERPINA, CYP genes are upregulated in adult liver.

	Fetal Liver				Adult liver
	10 weeks	**14weeks**	**18weeks**	**22weeks**	
No of genes	293	217	380	425	906

	10 week	14week	18 week	22 week	LiverAdult
TRANSFAC (Adjusted p value <0.05, human, sorted by Z-score) (Top 10)	GATA2	NFATC3	FOXC1	GATA2	FOXC1
	HNF4A	POU2F2	MEF2A	LTF	GATA2
	SMARCA2	MEF2A	GLI2	STAT3	CACYBP
			HOXD9	RUNX2	CEBPA
			NR5A2	PPARG	CEBPB
			RUNX1	NFIA	USF2
			YY1	FOXC1	CRTC3
			GATA2	SPI1	KLF11
			MZF1_1-4	ETS2	GATA1
			E2F1	NR5A2	JUN
			FOXL1	LEF1	KLF4
			KLF11	IKZF1	ZNF148
			POU2F1	FOXL1	NR3C1
			PITX2	MIR133B	NFIA
			POU1F1	CACYBP	PURA
			KLF4	FOS	NR5A2
			TFAP2A	POU2F1	POU2F2
			ELK1	ZBTB16	ETS1
			KLF13	CREB1	FOXL1
			USF2	NFKB1	HMGA1
			LEF1	POU2F2	PGR
					ATF2

Figure 3.6 Uniquely top 10 expressed transcriptional factors

Uniquely top 10 expressed transcriptional factors at each sates were represented in this table. At week 10 GATA2, HNF4a and SMARCA2 genes were expressed. At week 18 we have noticed the expression GLI2, FOXC1 **a critical regulator of hematopoietic stem cells**, HOXD9, E2F1, FOXL1 **hepatic progenitor bilineage potential gene** and LEF1. In week 22 we have noticed the expression of transcriptional factors such as FOXL1 a hepatic progenitor specific marker, PPARG, STAT3 involved in hepatocyte differentiation, GATA2, FOS and CREB genes. Interestingly we have noticed the of CEBPa/b, HNF4a, GATA1/2/3, SOX10 and HNF1a expression at adult liver validating that the liver maturation.

Functional clusters specific to week 10

Term	P-value
Negative regulation of hormone secretion (GO:0046888)	0.004430164
Blood circulation (GO:0008015)	0.005930383
glycosphingolipid metabolic process (GO:0006687)	0.009380439
Mismatch repair (GO:0006298)	0.010567764
Cell structure disassembly during apoptosis (GO:0006921)	0.011817883
Apoptotic mitochondrial changes (GO:0008637)	0.011817883
Regulation of MAPKKK cascade (GO:0043408)	0.012906145
Muscle contraction (GO:0006936)	0.013263597
Regulation of protein binding (GO:0043393)	0.014501581
Glycolipid metabolic process (GO:0006664)	0.014501581
Positive regulation of MAPKKK cascade (GO:0043410)	0.017421856
Small GTPase mediated signal transduction (GO:0007264)	0.018296539
Enzyme linked receptor protein signaling pathway (GO:0007167)	0.023441468

Functional clusters specific to week 14

Term	P-value
Glycosphingolipid metabolic process (GO:0006687)	0.004641597
Gamete generation (GO:0007276)	0.005061824
Spermatogenesis (GO:0007283)	0.003447177
Cellular cation homeostasis (GO:0030003)	0.009929767
Glycolipid metabolic process (GO:0006664)	0.007229052
Ion homeostasis (GO:0050801)	0.025551702
Cellular homeostasis (GO:0019725)	0.030550563
Sphingolipid metabolic process (GO:0006665)	0.031267963
Cellular ion homeostasis (GO:0006873)	0.020256173
Cellular metal ion homeostasis (GO:0006875)	0.027927012
Metal ion homeostasis (GO:0055065)	0.03008439
Membrane lipid metabolic process (GO:0006643)	0.038272107
Sodium ion transport (GO:0006814)	0.017964263
Hemostasis (GO:0007599)	0.034002368

Functional clusters specific to week 18

Term	P-value
Proteoglycan metabolic process (GO:0006029)	0.001885059
Establishment or maintenance of cell polarity (GO:0007163)	0.032936844
Glycoprotein metabolic process (GO:0009100)	0.013582578
Polysaccharide metabolic process (GO:0005976)	0.007053152
Nucleosome assembly (GO:0006334)	0.040934127
G2/M transition of mitotic cell cycle (GO:0000086)	0.015231583
Cell-cell junction organization (GO:0045216)	0.032936844
Cell-cell junction assembly (GO:0007043)	0.015231583

Functional clusters specific to week 22

Term	P-value
Cardiac muscle contraction (GO:0060048)	0.001158895
Steroid catabolic process (GO:0006706)	0.001158895

Positive regulation of T cell activation (GO:0050870)	0.001669975
Cardiac muscle tissue morphogenesis (GO:0055008)	0.002366931
Steroid metabolic process (GO:0008202)	0.002371525
Regulation of T cell activation (GO:0050863)	0.006008578
Striated muscle contraction (GO:0006941)	0.007286068
Lipid catabolic process (GO:0016042)	0.007358378
L-fucose catabolic process (GO:0042355)	0.010342888
Regulation of lymphocyte activation (GO:0051249)	0.012563201
Muscle contraction (GO:0006936)	0.014317794
Regulation of vasoconstriction (GO:0019229)	0.014396168
Positive regulation of adaptive immune response (GO:0002821)	0.014396168
Positive regulation of immune system process (GO:0002684)	0.014895464
Regulation of muscle contraction (GO:0006937)	0.015743508
Ventricular cardiac muscle morphogenesis (GO:0055010)	0.016639383
Fructose metabolic process (GO:0006004)	0.016639383
Membrane protein ectodomain proteolysis (GO:0006509)	0.016639383
Steroid biosynthetic process (GO:0006694)	0.016917487

Functional clusters specific to Adult liver

Term	P-value
Defense response (GO:0006952)	2.41E-06
Inflammatory response (GO:0006954)	3.30E-05
Cell-cell signaling (GO:0007267)	6.43E-05
Cell communication (GO:0007154)	4.56E-05
Hormone metabolic process (GO:0042445)	1.30E-04
Response to wounding (GO:0009611)	3.56E-04
Cell surface receptor linked signal transduction (GO:0007166)	5.66E-04
Response to external stimulus (GO:0009605)	7.61E-04
G-protein coupled receptor protein signaling pathway (GO:0007186)	8.01E-04
locomotory behavior (GO:0007626)	4.71E-04
Signal transduction (GO:0007165)	6.40E-04
Chemotaxis (GO:0006935)	7.31E-04
Anatomical structure development (GO:0048856)	9.29E-04
Potassium ion transport (GO:0006813)	5.42E-04
Cellular defense response (GO:0006968)	0.001336351

Figure 3.7 GO Pathway analysis

GO Pathway analysis with unique gene list: The functional groups from week 10 till week 18 involved in cell cycle, mRNA processing, ribosome activity, cellular homeostasis and MAPKKK regulation. In week 22 we have noticed were, mesoderm development, regulation of lymphocyte activation, metabolic process, embryonic development and Regulation of T cell activation.

Functional pathways analysis specific to week 10
Term
HSA04020 CALCIUM SIGNALING PATHWAY
HSA05040 HUNTINGTONS DISEASE

Functional pathways analysis specific to week 14
Term
HSA00603 GLYCOSPHINGOLIPID BIOSYNTHESIS GLOBOSERIES
HSA04540 GAP JUNCTION
HSA04916 MELANOGENESIS
HSA01430 CELL COMMUNICATION
HSA04720 LONG TERM POTENTIATION

Functional pathways analysis specific to week 18
Term
HSA05217 BASAL CELL CARCINOMA
HSA05222 SMALL CELL LUNG CANCER
HSA04340 **HEDGEHOG SIGNALING PATHWAY**

Functional pathways analysis specific to week 22
Term
HSA04740 OLFACTORY TRANSDUCTION
HSA04640 HEMATOPOIETIC CELL LINEAGE
HSA04660 T CELL RECEPTOR SIGNALING PATHWAY
HSA04940 TYPE I DIABETES MELLITUS
HSA04912 GNRH SIGNALING PATHWAY
HSA04662 B CELL RECEPTOR SIGNALING PATHWAY
HSA04340 HEDGEHOG SIGNALING PATHWAY
HSA04650 NATURAL KILLER CELL MEDIATED CYTOTOXICITY
HSA05010 ALZHEIMERS DISEASE
HSA04614 RENIN ANGIOTENSIN SYSTEM

Functional pathways analysis specific to adult liver
Term
HSA00040 PENTOSE AND GLUCURONATE INTERCONVERSIONS
HSA00980 METABOLISM OF XENOBIOTICS BY CYTOCHROME P450
HSA00150 ANDROGEN AND ESTROGEN METABOLISM
HSA00500 STARCH AND SUCROSE METABOLISM
HSA00860 PORPHYRIN AND CHLOROPHYLL METABOLISM
HSA04060 CYTOKINE CYTOKINE RECEPTOR INTERACTION
HSA00120 BILE ACID BIOSYNTHESIS
HSA00232 CAFFEINE METABOLISM

Figure 3.8 KEGG Pathway analysis

KEGG Pathway analysis with unique gene list: The KEGG pathway analysis involve in week 10 calcium signaling pathway, in week 14 cell communication, gap junction and long-term potentiation. In week 18 we observe hedgehog signaling pathway and in week 22 hematopoietic cell lineage, hedgehog signaling pathway and renin angiotensin system.

Gene_ID	Gene Symbol	Gene Name	Liver(RPKM)
ENSG00000173432	SAA1	serum amyloid A1	21500
ENSG00000163631	ALB	albumin	16853
ENSG00000171564	FGB	fibrinogen beta chain	11432
ENSG00000171560	FGA	fibrinogen alpha chain	11051
ENSG00000197249	SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	10810
ENSG00000132693	CRP	C-reactive protein, pentraxin-related	10023
ENSG00000229314	ORM1	orosomucoid 1	8359
ENSG00000171557	FGG	fibrinogen gamma chain	6950
ENSG00000134339	SAA2	serum amyloid A2	3759
ENSG00000110245	APOC3	apolipoprotein C-III	2733

		Fetal Liver				Adult liver			
Gene Symbol	Gene Name	FL10w	FL14w	FL18w	FL22w	Ambio	Clonte	CMN1	CMN2
ALB	albumin								
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase	2.42	3.37	3.69	1.85			4.33	
APOA2	apolipoprotein A-II	1.33	1.24	1.18	0.53	3.45	1.92	1.98	1.69
RBP4	retinol binding protein 4, plasma	0.98	0.89	1.13	0.85	2.98	1.32	1.39	2.30
AHSG	alpha-2-HS-glycoprotein	2.42	3.01	1.24	0.52	1.86	0.72	1.64	1.55
FABP1	fatty acid binding protein 1, liver	4.27	3.29	4.14	1.77			3.75	3.20
CP	ceruloplasmin (ferroxidase)	0.50	0.81	0.65	0.39	0.85	2.68	2.17	2.53
ANGPTL3	angiopoietin-like 3	0.59	0.55	0.68	0.51	0.13	0.58	0.60	0.86
SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), mer	0.73	0.51	0.52	0.61	0.89	0.29	0.53	0.81
OIT3	oncoprotein induced transcript 3	1.93	1.62	1.36	1.52	2.43	2.19	2.35	2.70

Figure 3.9 Canonical pathway analyses

Canonical pathway analyses for liver specific gene list from public databases was compared with our data. The data shows that Albumin and SERPINA was the common gene that was upregulated across all stages of fetal liver and adult liver.

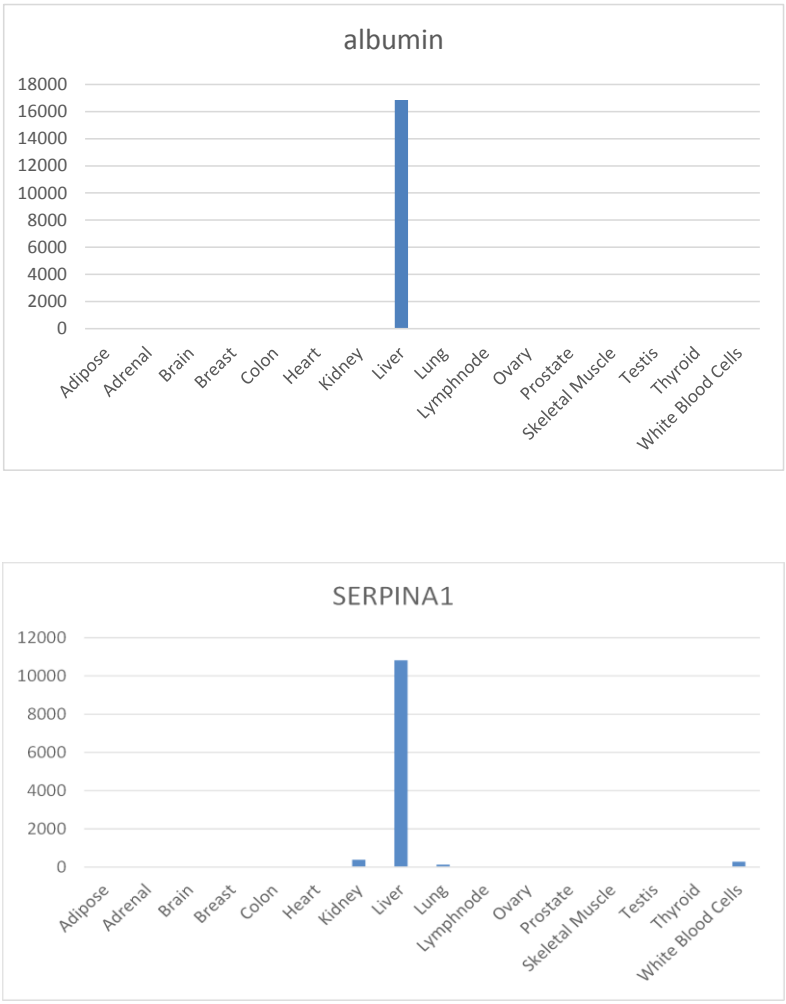
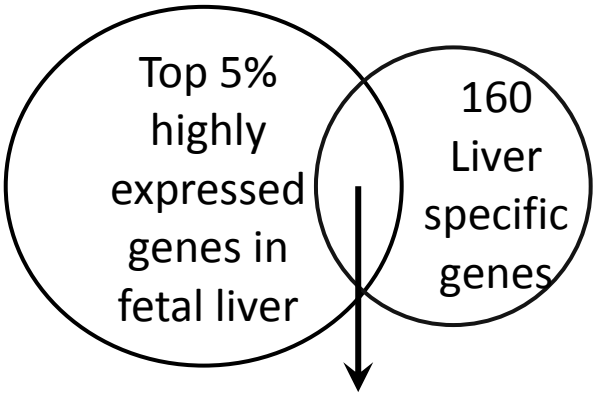


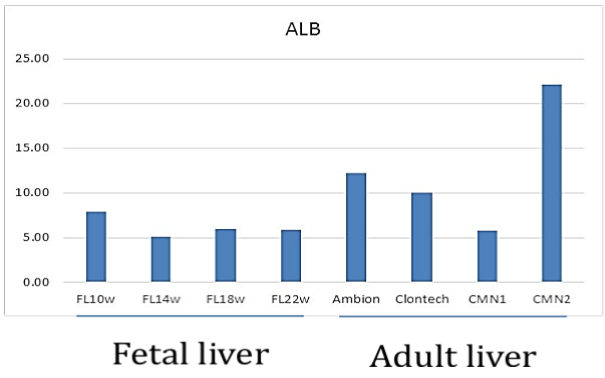
Figure 3.10 Albumin and Serpina liver specifc markers

Albumin and Serpina liver specifc markers were upregulated in adult liver. These two genes are common genes that were referred as a matured liver marker**.**

A



B



C

		Fetal Liver				Adult liver			
Gene Symbol	Gene Name	FL10w	FL14w	FL18w	FL22w	Ambio	Clonte	CMN1	CMN2
ORM1	orosomucoid 1	0.03	0.08	0.07	0.09				
HPX	hemopexin	0.01	0.06	0.06	0.04			0.67	0.65
APOC2	apolipoprotein C-II	0.09	0.01	0.14	0.24			0.54	
APCS	amyloid P component, serum	0.00	0.00	0.00	0.06				
ORM2	orosomucoid 2	0.03	0.04	0.15	0.14				
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	0.00	0.00	0.01	0.00				
F2	coagulation factor II (thrombin)	0.20	0.19	0.34	0.22				
AZGP1	alpha-2-glycoprotein 1, zinc-binding	0.09	0.10	0.24	0.30				
ITIH1	inter-alpha-trypsin inhibitor heavy chain 1	0.20	0.21	0.28	0.15	0.67	0.59		
HRG	histidine-rich glycoprotein	0.07	0.14	0.03	0.06				
PLG	plasminogen	0.20	0.12	0.17	0.15		0.66	0.64	
DHODH	dihydroorotate dehydrogenase (quinone)	0.35	0.21	0.36	0.43				

Figure 3.11 Top 5 % of liver specific marker

A. Top 5 % of liver specific marker from next generation sequencing. B. Albumin expressed both in fetal and adult liver. C. ORM1 and APOC2 genes specific for adult liver.

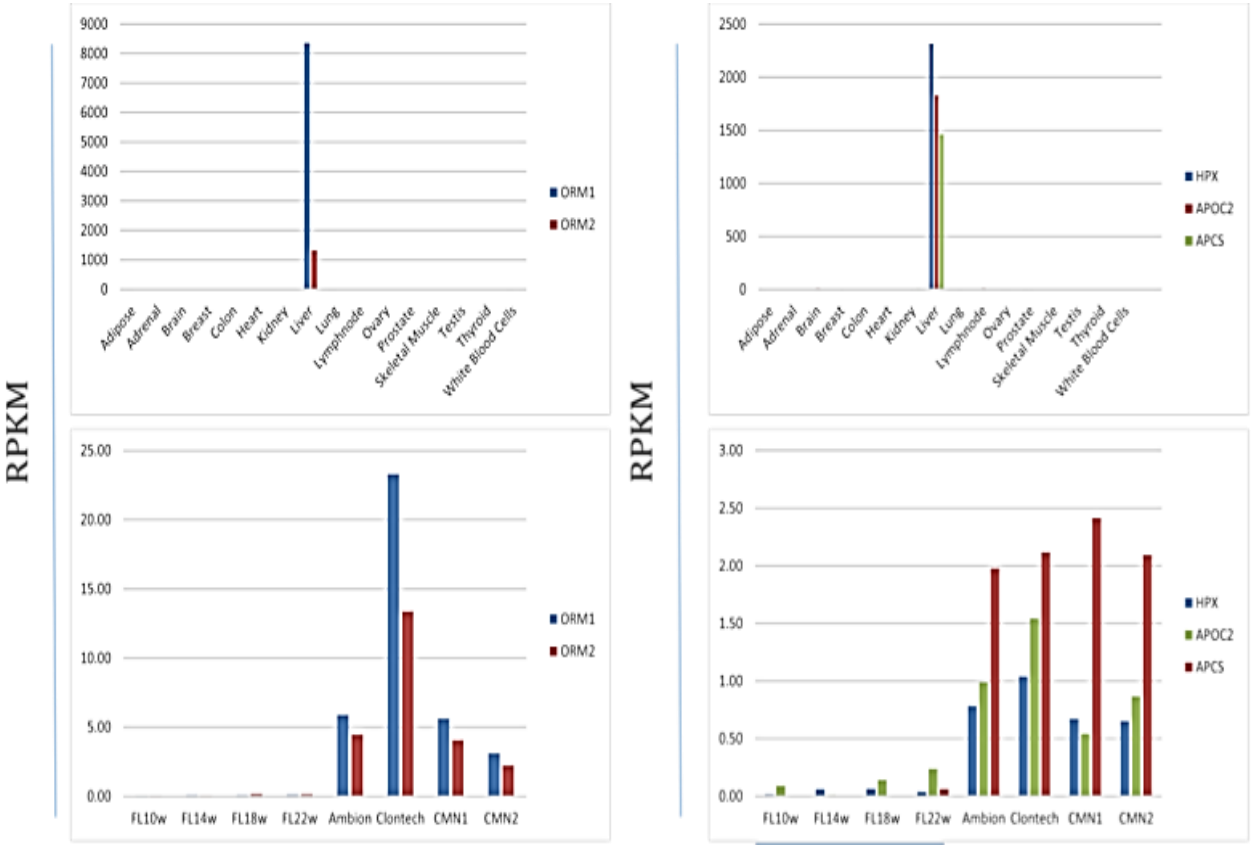


Figure 3.12 A. ORM1, APC5 and APOC2 genes expression

Next generation sequencing data shows the upregulation adult liver specific ORM1, APC5 and APOC2 genes specific for adult liver and down regulated in fetal liver.

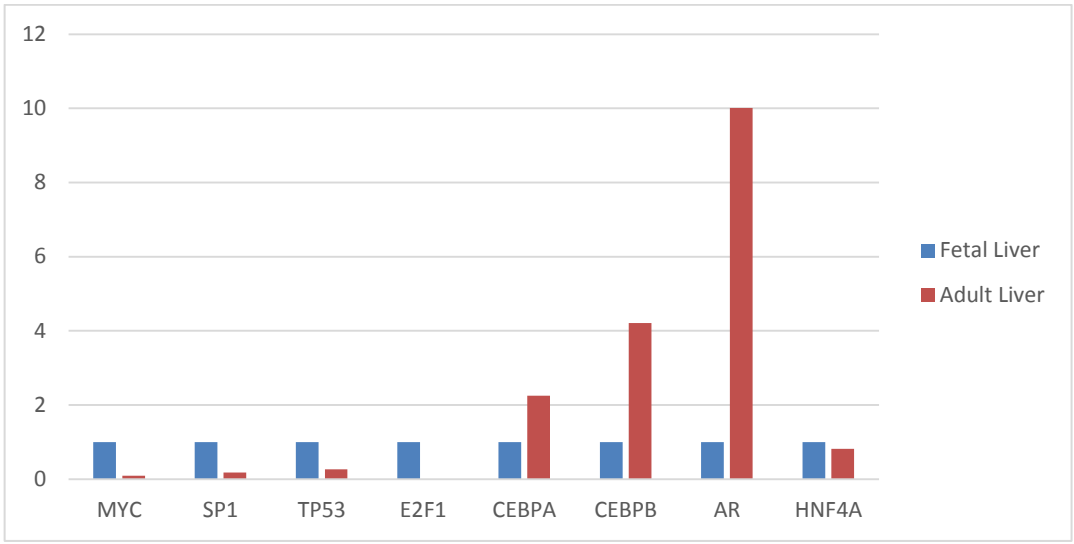


Figure 3.13 ORM1, APC5 and APOC2 gene expression. A. next generation sequencing data shows the upregulation adult liver specific ORM1, APC5 and APOC2 genes specific for adult liver and down regulated in fetal liver.

Chapter 4

in vitro Validation of genetic regulators of liver development

4.1 Background

The major hurdles that have limited the use of human hepatocytes in translational medicine, is the lack of a continual and reliable source of cells and the technical difficulty in maintaining their differentiated hepatocytic functions *in vitro* for significant periods of time (Leffert et al., 1978). Following liver injury, the human hepatocyte exhibits tremendous intrinsic ability to proliferate and regenerate itself *in vivo* (Fausto, 2004). However, despite the rapid advances in understanding of the liver anatomy and physiology, it has not been possible to proliferate human hepatocytes reliably in culture or prevent them from dedifferentiation and losing their hepatocytic functions.

As a result of these limitations, there have been significant efforts to identify and utilize alternative sources of cells that can be expanded easily in culture, and subsequently manipulated to give rise to hepatocytes, either by directed differentiation (hepatocyte linage stem/ progenitor cells) or transdifferentiation (stem cells from other lineages) (Dan and Yeoh, 2008). Candidate cells that have been reported to have this potential include embryonic stem cells, hematopoietic stem cells, mesenchymal stem cells (MSC) or mesenchymal lineage stem cells, hematopoietic stem cells, fetal liver progenitor cells adult liver progenitor cells or more recently, induced pluripotent stem cells or reprogrammed somatic cells (Dan and Yeoh, 2008).

Induced pluripotent stem cells or reprogrammed somatic cells are the two leading candidates progenitor cells being researched intensively to produce functional hepatocytes. While they can, in theory, be easily scaled up and ever improving

and more efficient hepatocyte differentiation protocols are being reported, the current challenges that stand in the way of clinical translation are the ability to expand these cells to clinically meaningful numbers as well as their differentiation into fully functional hepatocytes. Gene array studies of differentiated hepatocytes from these cells sources show that they are probably still at the stage of fetal hepatic side and are quite distant from the full metabolic capabilities of adult hepatocytes.

To overcome these challenges, we felt that identification and thorough understanding of the regulatory factors that occur in various stages of the physiological fetal liver development would be extremely useful.

Our overall aim was thus to study the human fetal liver development to identify the key mechanisms and potential target genes that can be applied in vitro for the expansion, maintenance and maturation of hepatocytes. The results from our array data were classified into three categories at each stage of fetal liver development are based on their upregulation: (i) Transcriptional factors, (ii) Growth factors and (iii) Extracellular matrix (described in detail in chapter 3).

This chapter summarizes the proof of principle in manipulating genes identified in our study *in vitro* to achieve the intended effect on liver progenitor cells, specifically for hepatocyte growth and maintenance.

Growth factors, extracellular matrix and signaling factors that are significantly up regulated at each specific phase, would likely be the putative factors that play a key role in the regulation of that phase.

Based on our observation and gene clustering, we identified different phases in liver development. We chose the genes based on these classifications. Week 10-11 Specification phase - genes at this stage were mostly embryonic stem cell factors and would be useful for *in vitro* iPSC manipulation. Week 14-15: Expansion / differentiation phase - Genes at this stage were extracellular matrix proteins Collagen, Laminin and Hyaluronic acid. Week 17-19: Hematopoiesis and Proliferation Week 20-24: Maturation, EMT phase - genes that were upregulated at this stage were SHH signaling, FGF, FGFR and CTGF. Adult Liver: functional.

Specifically, week 11 to 14 would represent the phase of hepatoblast playing the role of a transit-amplifying cell in expansion and differentiation into hepatic lineage. Similarly, week 19 to 22 would represent the phase of progenitor cell proliferation by mesenchymal epithelial transition. Week 23 to adult stage would represent the phase of hepatocyte maturation.

Using this assumption, we identified the following genes from the various classes of extracellular matrix, growth factors and signaling factors that are significantly up or down regulated **(Figure 4.1)**. We then tested these factors in *in vitro* cultures as modifiable variables in the microenvironment of fetal liver epithelial cells to validate the effects.

Table 6: Genetic regulators used in *in vitro* experiment

ECM	Collagen, Lamanin and Hyaluronic acid
Growth Factors	FGF, FGFR and CTGF

4.2 Materials and methods

4.2.1 Processing of fetal liver cells

Methods for the isolation and processing of fetal liver cells were modified from previously reported protocols (Dan et al., 2006). Fetal liver collected on time of call from the hospital range from 10 weeks to 24 weeks of fetal mid-gestational stage. Fetal livers were aseptically dissected from the human fetuses under hospital environment and maintained in basal medium on ice until use. They are transferred to basal medium for transport. To extract fetal liver cells, fetal livers were treated with 0.3% collegenase for 15 minutes at 37^{o}. Subsequently, the livers were triturated with P1000 pipettes to achieve a single cell suspension, and 10% FBS-containing medium was added to neutralize the collegenase. The cells were pelleted at 700 rpm for 5 minutes, resuspended in the SEGELENS buffer, and re-pelleted at 1000 rpm for 3 minutes. The cell pellet was resuspended in 1 ml of culture medium, and filtered with a cell strainer (40μm pore size). Another ml of medium was used to rinse the filter and combined with the cell suspension. In average, this process yields $2X10^{6}$ fetal liver cells in according to the gestational age of the fetus **(n = 3).**

4.2.2 Culture of fetal liver cells

The compositions of the culture media studied in this is described in Table 12 incomplete culture media (DMEM), which were used for the transport of fetal liver from KK women and children hospital to the lab; refer to media that were not supplemented with insulin, EGF, HGF and OSM. Complete culture media included all those factors and were used for the rest of the culture period.

Table 7: Formulations of the culture media

Complete medium	**Concentration**	**Final volume**	
William's E medium (basal medium)		450 ml	Gibco
Nicotinamide	10mmol/ml	5 ml	Gibco
HEPES	20mmol/ml	10 ml	Gibco
Dexamethasone	100umol/ml	1 ml	Sigma-Aldrich
ITS+ premix	6.25ug/ml	5ml	BD Biosciences
Sodium bicarbonate	17mmol/ml	10 ml	Gibco
Sodium pyruvate	550mg/ml	5 ml	Gibco
L glutamine	2 mol/ml	5ml	Gibco
Ascorbic Acid	0.2 mmol/ml	5 ml	Sigma-Aldrich
Glucose	14 mmol/ml	12.6 ml	Sigma-Aldrich
Gentamicin (strep/pen)	50ug/ml (5ml)	5ml	Sigma-Aldrich
EGF	20ng/ml	0.5ml	BD Biosciences
FBS	100%	10%	Sigma-Aldrich

4.2.3 Coating culture plates: On day 0 (before the day of culture set-up), collagen-working solution was prepared by diluting the stock (3mg/ml) 1:3 with sterile water. Tissue culture treated polystyrene plates were coated with the

collagen working solution at room temperature for overnight in BSII cabinet and the next date rinsed with PBS. The wells are stored in 4 degrees until future use.

The processed fetal liver cells were counted by Trypan Blue exclusion, and diluted with the complete culture medium of interest to $2X10^5$cells/ml. This suspension was plated onto the collagen-coated plates at desire volume according to the plate size, resulting in a plating density of $5X10^4$cells/cm^2. After 4 hours of culture, culture supernatant was removed and the culture wells were rinsed with PBS to remove non-adherent cells. Complete culture medium was then added to the well. Culture medium was changed every 2 – 3 days thereafter.

4.2.4 Determining ideal Culture Conditions extra cellular matrix

To test the ideal ECM obtained for the array data for fetal liver cultures, extracellular matrix substrates were studied. Plates were coated with laminin (5ug/ml), collagen (1:5), fibronectin (5ug/ml), gelatin (5ug/ml) and hyaluronic acid (Suplasyn, Bioniche Life Sciences Inc., London, Ontario, Canada), washed with PBS and air-dried in culture hood over night before use.

4.2.5 In Vitro Differentiation Protocol

All differentiation protocols were performed in triplicate with freshly isolated human fetal liver cells. Basic culture medium was purchased from Gibco / Invitrogen (Carlsbard, CA) and supplements were from Sigma Aldrich (St Louis, MO).

4.2.6 Hepatocyte Differentiation

Cultures were treated with standard hepatocyte culture medium CSHFM (Complete described in table 12) and Oncostatin M (OSM) (10ng/mL; R&D Systems, Minneapolis, MN) was added as another extra supplement which has been proven to differentiate into functional hepatocytic cells.

4.2.8 Assessment of cell proliferation

Cell growth was monitored by alamar blue assay. The assay is based on the ability of viable, metabolically active cells to intracellular reduce reassuring to resorufin and dihydro- resorufin. The reduction enters cytosol and is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH. It's then converted to the oxidized (or non-fluorescent) blue form to the reduced (fluorescent) red form.

To perform the alamar blue assay, culture media were removed and the wells were rinsed with PBS to remove residual media with phenol red. Subsequently, 1 ml of alamar blue diluted with OPTI MEM medium was added to each well. . The plate was placed in a cell culture incubator for 1 hour. Afterwards, 200µl of suspension (in triplicate) from each culture well was transferred to a 96-well plate. Optical absorbance was measured in dual mode (at 540 nm with a reference at 630 nm) with an ELx808 micro plate reader (BIO-TEK Instruments, Winooski, VT).

4.2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is the abbreviation of enzyme-linked immunosorbent assay. It is a useful and powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine, sperm and culture supernatant. The basic principle of an ELISA is to use an enzyme to detect the binding of antigen (Ag) antibody (Ab). The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Ags or Abs in a sample, depending on how the test is designed.

Human albumin concentrations from culture media were measured by the sandwich enzyme-linked immuno-sorbent assay as previously described. The capture antibody (goat anti-human antibody; 4 mg/mL), the detection antibody (peroxidase-conjugated goat immunoglobulin G fraction to human albumin; 3 mg/mL), and the purified human albumin standards were purchased from ICN Pharmaceuticals (Durham, NC).

Results:

4.3 To evaluate the suitable microenvironment for proliferation human fetal liver cells:

From the array data we identified specific ECM proteins were highly unregulated at week 14 compared to 10-week fetal liver sample. Here we would like to mention that the 14-week stage was associated with hepatoblast proliferation, in which we observed the upregulation in the expression profile of laminin3,

collagen A2 as well as hyaluronic acid. The expression was significantly reduced in the later stages of liver development. Although we have identified these specific genes isoforms, we were restricted to use the commercially available isoform of these genes for our research. This limitation of unavailability for extracellular matrix is one of the hurdles in defining the microenvironment i*n vitro*. We tested each of these ECM individually and in combination to determine their effect on fetal hepatoblast culture.

To understand the mechanism of action of these variables in *in vitro* cultures, we made careful distinction between the effects of these variables on cell expansion and morphology.

4.3.1 Proliferation: Which is the best Extracellular matrix?

Cell proliferation was determined by using alamar blue assay. Collagen and Collagen/Laminin has the highest adherence but does not promote proliferation. Laminin, in combination with collagen, and/or HA, resulted in the highest proliferation of fetal liver cells **Figure 4.2**.

4.3.2 Effect on expansion

The cells cultured on collagen only resulted in maintenance of fetal hepatoblast morphology. In collagen coated plate, we also observed cells hepatocytes were binuclear, which is a typical characteristic of hepatocytes morphology *in vivo*. The cells cultured on a combination of collagen, laminin and Hyaluronic acid maintained the immunophenotype but resulted in spindle-type morphology (**Figure 4.3**). The other combinations of these ECM were not as effective as

Collagen coated plates in maintaining its phenotype. To confirm maintenance of cell immunophenotype, we compared morphology with EPCAM and CD44 immunostaining **(Figure 4.4)**.

From the results, we conclude that, using combinations of laminin and collagen maintains the morphology and proliferation for at least 2 passages.

4.4 Growth factors

Growth factors are essential component in every cell culture practice. Every liver research group is probing to find a right combination of growth factors to maintain, differentiate and expand the hepatocytes *in vitro*. We are no exceptions and from our array analysis we have noticed the upregulation of signaling pathways such as FGF and CTGF at week 22 compared to week 10. Our hypothesis was to study these signaling factors, which were upregulated at week 22 (Maturation, EMT phase) in the maintenance and proliferation of liver cells *ex vivo*.

4.4.1 FGF growth factor signaling

In our qPCR-array data, the expression of FGF signaling was analyzed by comparing the fetal liver stages and adult liver against 10-week as control. The expression of FGF1 was upregulated at 14 weeks s and was down regulated in the adult liver, suggesting its importance in the early developmental stages. In contrast, the expression of FGF2 was upregulated in adult liver and was down regulated in fetal stages. We also observed the expression of FGFR1 which was down regulated in adult liver may be associated with matured liver (**Figure 4.5**).

We hypothesize that 1) FGF1 may have a role on cell proliferation because it was upregulated at 14 weeks. 2) In addition, FGFR1 and FGFR2 receptors downregulated at adult liver may have a role in expansion and differentiation.

Fibroblast developmental factors (FGFs) signaling from the cardiac mesoderm induces the liver in the ventral foregut endoderm. During the time of hepatogenesis, the cardiogenic mesoderm expresses at least three out of the eighteen known FGFs, and the ventral foregut endoderm expresses at least two of the four tyrosine kinase FGF receptors (Jung et al., 1999). During the time of hepatic induction, the endoderm distinctively expresses FGF receptor 4 (FGFR4) (Stark et al., 1991)) and both the endoderm and cardiac mesoderm express FGF receptor 1 (FGFR1) (Sugi and Lough, 1995). In situ immunohistochemistry studies indicated that FGF1 and FGF2 are generally induced in the cardiac mesoderm at the 7-8 somite phases in mouse (Jung et al., 1999). Purified FGF1 and FGF2 were each found to effectively induce early liver-specific genes within the ventral foregut endoderm, when the endoderm was isolated from 2-6 somite stages. Studies have demonstrated that FGFs and BMPs are effective in mediating early hepatic differentiation. HGF supports mid-late hepatic phenotype (e.g. ALB expression) (Kumashiro et al., 2005), but does not induce functional maturation. Stepwise supplement of FGF, HGF and a combination of insulin-transferrin-sodium selenite (ITS), dexamethasone and OSM was successful.

FGF signaling is one of the most important signaling pathways studied in liver development. It is important to mention that FGF growth factors such as FGF1

and FGF2 have been already determined to be useful in culture medium to maintain liver cells.

The proliferative effect of FGF1 and FGF2 Growth factors on fetal liver culture was assessed *in vitro* by alamar blue staining. Unfortunately, the *in vitro* effects of FGF1 and FGF2 were not congruous with the regulation pattern with our RT-PCR.

What was consistent however was that, upon addition of anti FGFR1 and anti FGFR2 to block the FGFR1 and FGFR2 receptors in culture, there was significant proliferation of liver cells after one month. FGF signaling of the various isoforms is believed to be a complex interplay between the isoforms and its receptors. In our case, the ratio of FGF and FGFR1 mediates hepatoblast proliferation at 14 weeks. This effect is likely complex and does not avail the potential for FGF manipulation for ex-vivo expansion or maturation of hepatocytes (**Figure 4.6 and Figure 4.7**).

4.5 CTGF growth factor

In our array data we noticed the significant upregulation of CTGF growth factor at week 22 fetal liver compared to week 10 **(Figure 4.8)**. Previous studies have reported that CTGF (Connective tissue Growth Factor) has a variety of functions in developmental biology, which includes angiogenesis, cell adhesion, proliferation, skeletal development, tooth development, and apoptosis (Brigstock, 2003; Perbal et al., 2003; Rachfal and Brigstock, 2005). CTGF has been shown to stimulate extra cellular matric (ECM) along with mitogenic and chemotactic

activities. This indicates that CTGF acts as a central driver in cartilage/bone growth and regeneration (Heng et al., 2006; Kanaan et al., 2006; Kubota and Takigawa, 2007; Ono et al., 2007). The importance of CTGF in skeletal development is demonstrated with the generation of CTGF knockout mice (Yamaai et al., 2005). CTGF ablation results in mice with mishappened skeleton, craniofacial abnormalities, and defects associated with endochondral ossification, and attributes to defects in cell proliferation, matrix formation, and remodeling during endochondral ossification. However, the role for CTGF in developmental biology of liver is unknown. Our hypothesis is to analyze the importance of this growth factor that express at maturation phase week 22 in *in vitro* liver cell culture.

Effect of CTGF growth factor on human fetal liver culture was studied by adding commercially available recombinant CTGF into culture medium. The result show that the addition of CTGF has effect on cell proliferation, which has increased two folds compared to control without growth factors (**Figure 4.9 and Figure 4.10).** In addition to the proliferation effect of the fetal liver cells, we observed that the CTGF had no effect on maturation of the cells. The protein assay showed the production of serum albumin no increase compared to control cells, suggesting that CTGF drives mainly proliferation but not differentiation.

Limitations and future studies:

The unavailability of the desired extracellular matrix isoforms – in the future studies we need to analyze the effect of these observed isoforms in maintaining the liver cells.

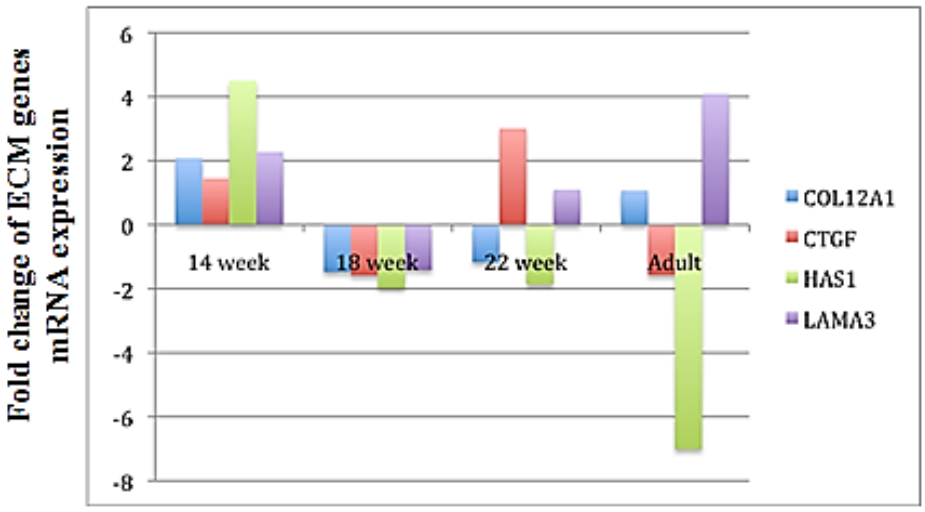


Figure 4.1 Key extracellular matrix in liver development.

mRNA-expression levels of ECM proteins Collagen, laminin, and HA were measured by real-time-PCR experiment. cDNA was converted from first strand cDNA kit. qRT-PCR data shows the upregulation of COL12A1, CTGF, HAS1 and LAMA3 upregulated at week 14.

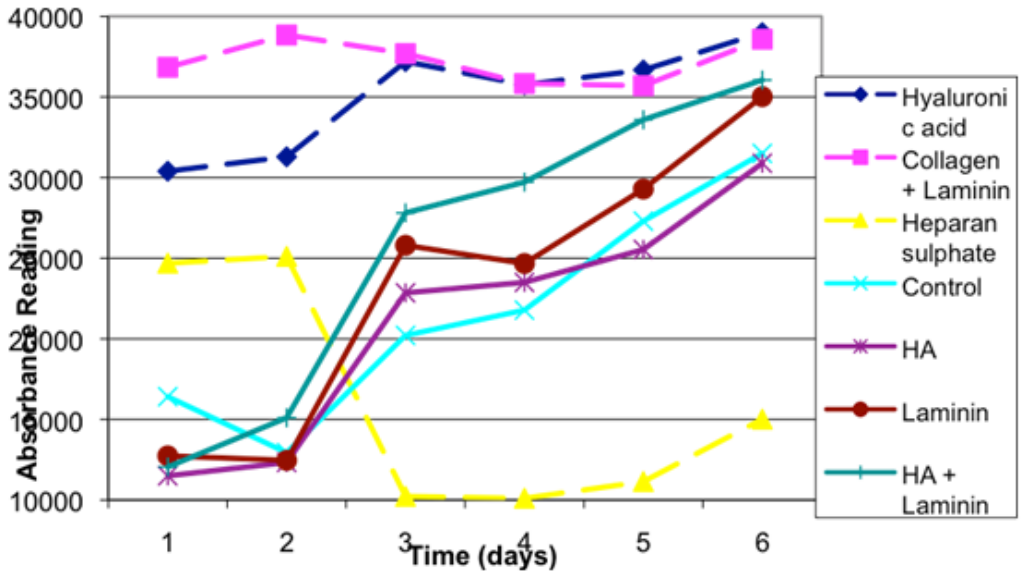


Figure 4.2 Cell proliferation in each well was measured by Alamar Blue dye reduction.

Fetal liver cells were plated in culture medium containing 10% FBS with different feeder layers. Collagen, lamanin and hyaluronic acid ECM were commercially purchased coated on to the pates. The cells were maintained for 6 days. The cells coated with collagen lamanin were maintained morphologically and functionally. With other combinations of EMS the proliferation reduced in time with culture. The control cells were coated with collagen only.

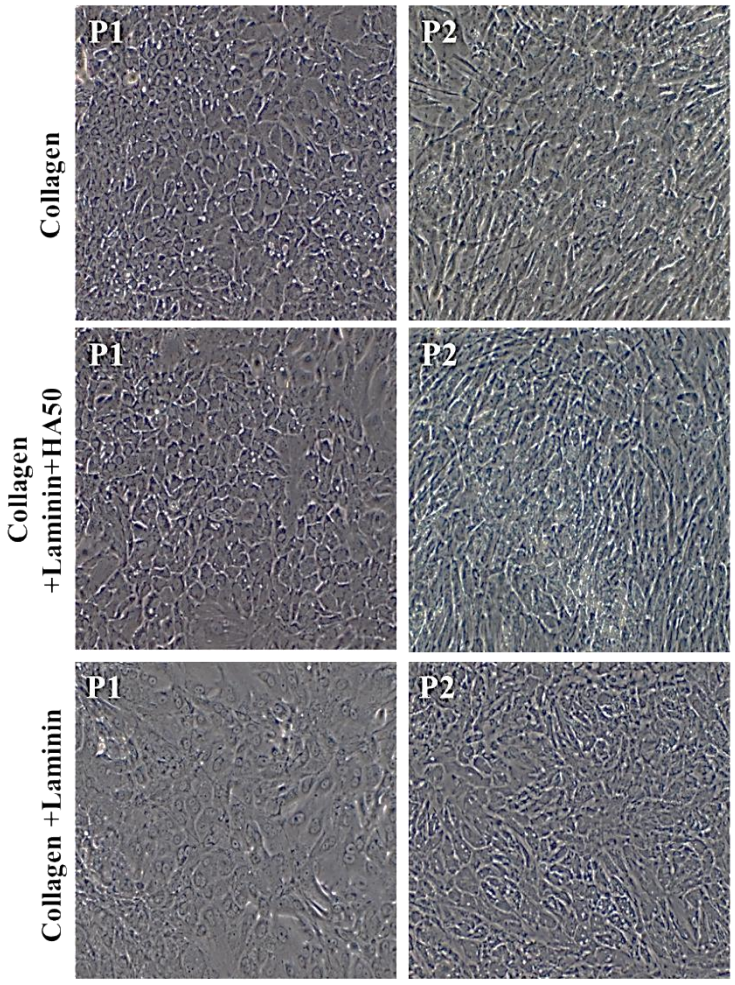


Figure 4.3 Extra Cellular matrix.

Phase contrast images show the morphological changes of fetal liver cells in vitro. Fetal liver cells were cultured on different culture conditions for up to two passages. A) Collagen B) Combination of HA450, Laminin and Collagen C) Collagen and laminin. Cells on collagen plated were maintained morphologically even after 2 passages. Combinations with Collagen, laminin and HA50 showed morphologically differences and phenotypic changes.

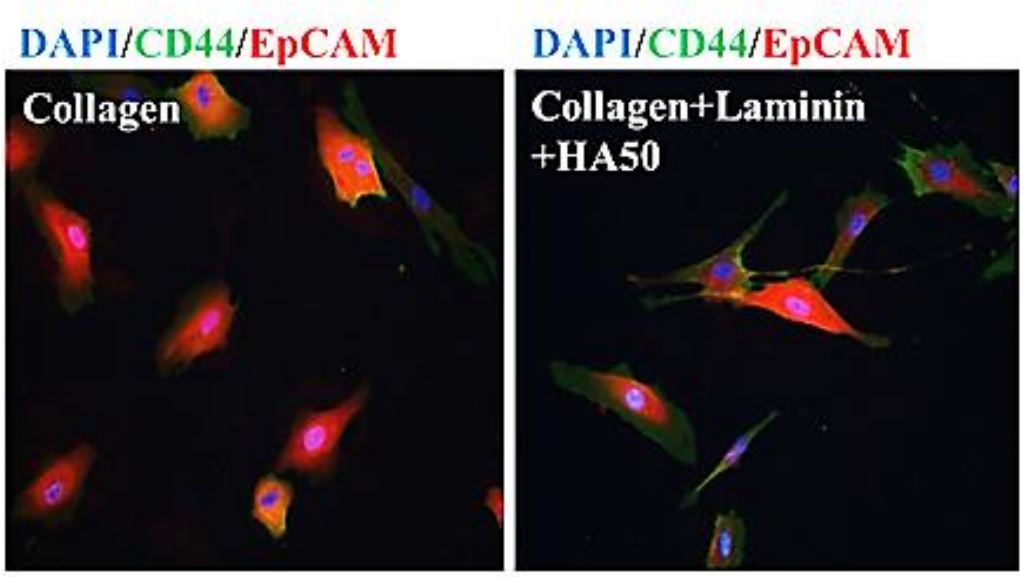


Figure 4.4 Phenotypic maintenance

Immunophenotype of CD44 (green) and Epcam (red) on liver cells cultured for 15 days in culture. Collagen+lamanin+hyaluronic acid did main the immunophenotyping of the fetal cells but the cell morphology was lost in contrast Collagen alone retain the both morphology and phenotype.

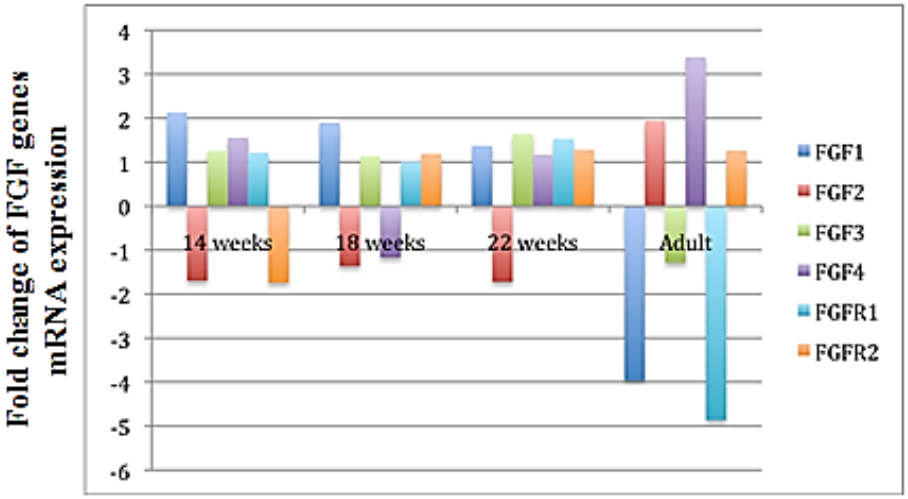


Figure 4.5 mRNA-expression levels of FGF signaling pathway was measured

FGF1 was upregulated at 14 weeks suggesting its importance in hepatoblast proliferation but was down regulated during adult liver. In contrast, the expression of FGF2 was upregulated in adult liver and was down regulated in fetal stages. We also observed the expression of FGFR1 which was downregulated may be associated with matured liver.

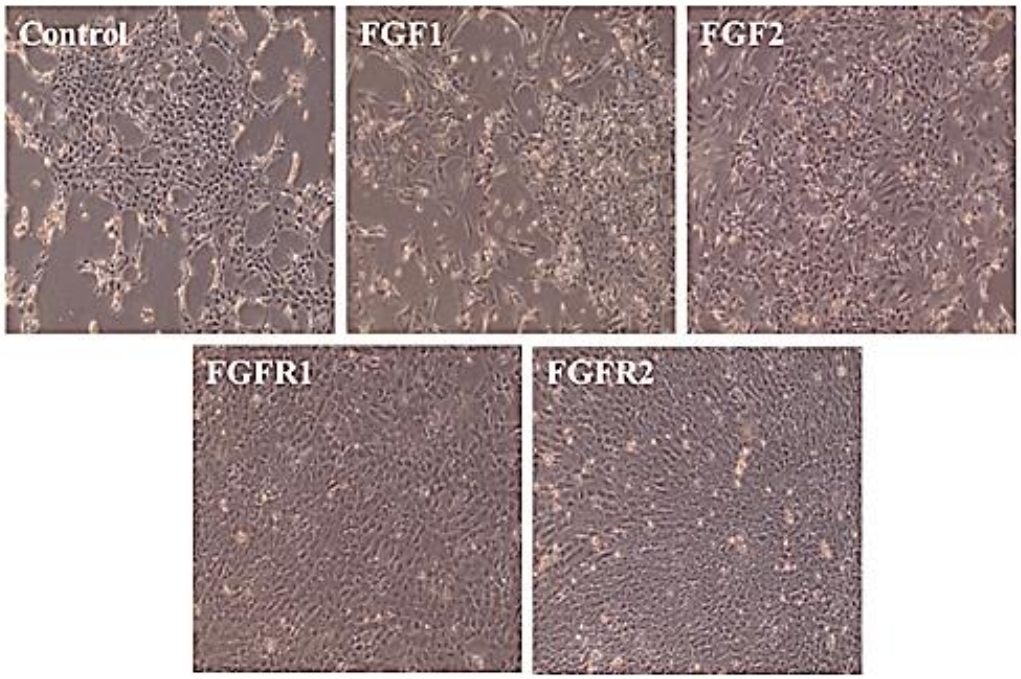


Figure 4.6 Pathway inhibitions by monoclonal antibodies.

Phase contrast images show the morphological changes of fetal liver cells in vitro. After one month in culture, we observed blocking the FGFR receptors maintain the expansion and proliferation of fetal liver cells, whereas addition of FGF1 and FGF2 were not effective compared to control.

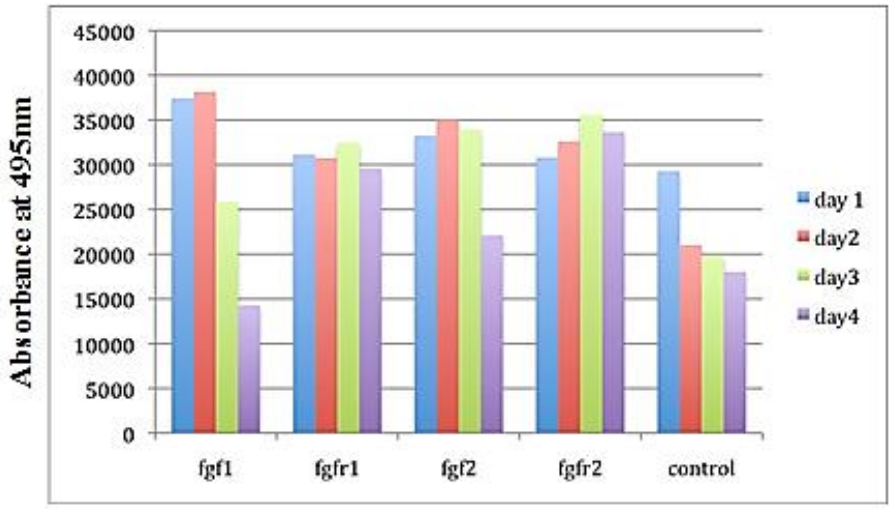


Figure 4.7 Cell Proliferation Assay.

Cell proliferation in each well was measured by Alamar Blue dye reduction. FGFR1 and FGFR2 maintained the cells while FGF1 reduce in time with culture compared to control cells.

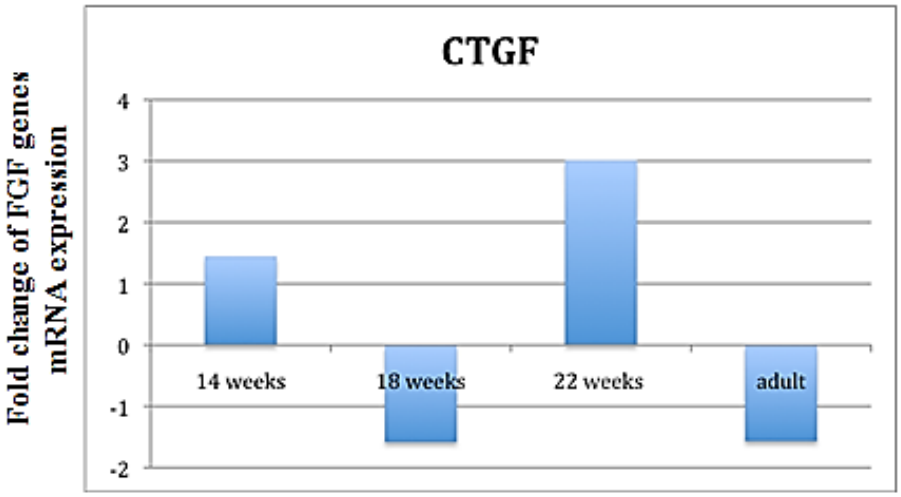


Figure 4.8 mRNA-expression levels of CTGF signaling were measured.

mRNA expression levels of CTGF was measured. CTGF was upregulated at week 22.

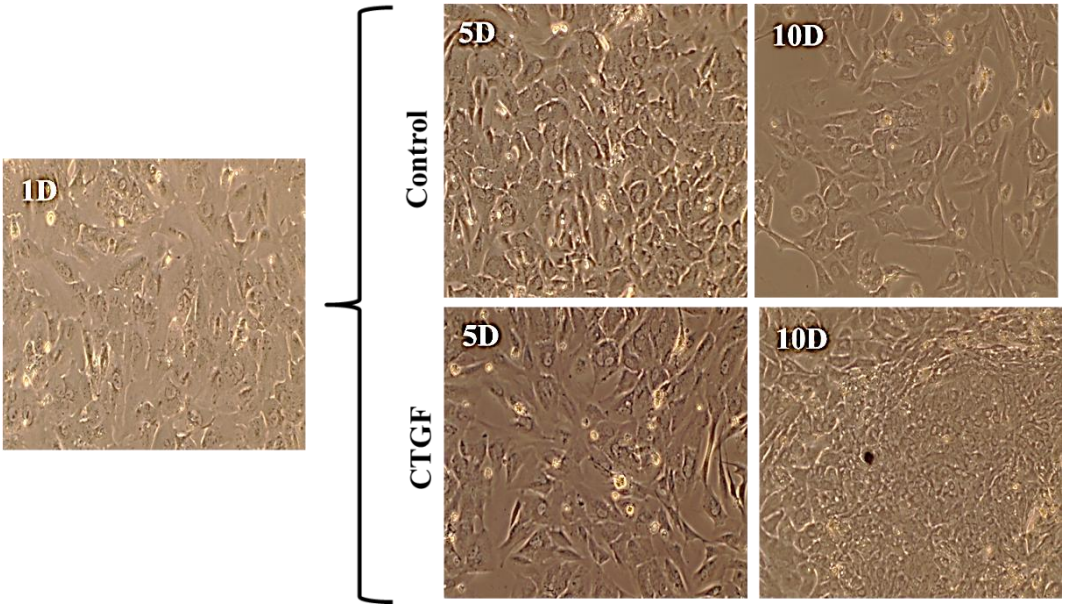


Figure 4.9 CTGF effect

Phase contrast images show the morphological changes of fetal liver cells in vitro. CTGF gene maintains and proliferate liver cells in culture

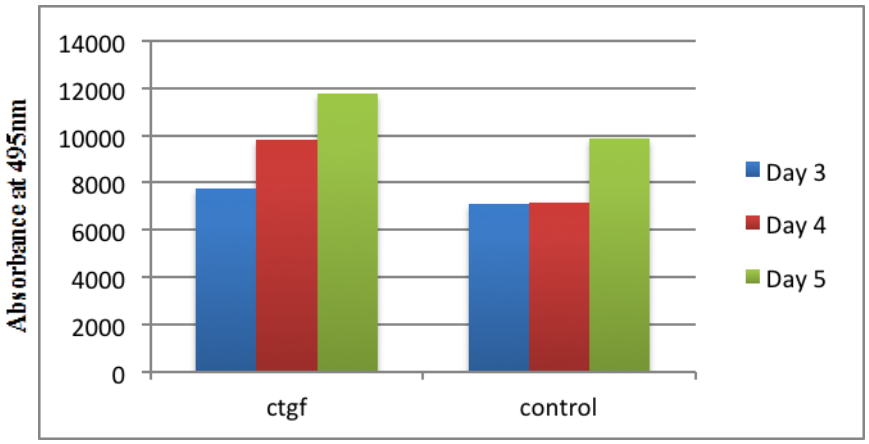


Figure 4.10 Cell Proliferation Assay.

Cell proliferation in each well was measured by Alamar Blue dye reduction. A) CTGF recombinant protein addition shows reduction in proliferation Vs. Control. CTGF Growth factor has no effect on cell proliferation.

Chapter 5

Correlating genetic determinants of liver progenitor cell proliferation in vivo

5.1 Background

In our previous chapters, we have elaborated on identifying key transition phases of fetal liver development. From our genetic studies, we were able to identify key genes that were up regulated at various stages of fetal liver development. We further showed that these factors were instrumental in driving and regulating hepatoblast proliferation and expansion *in vitro*. To further validate the importance and relevance of these factors, we explored if they were also involved in *in vivo* systems where the ontogeny of liver and its lineages is recapitulated.

The current model that can replicate the liver progenitor proliferation besides the fetal liver is the cirrhotic liver model. This is supported by the work done by Lola Reid (Zhang et al., 2008), and has shown that the progenitor cell proliferation in liver cirrhosis is symmetrical to fetal liver development. In that study, it was described that the liver regenerative process is parallel to those occurring in development and involve populations of stem cells and progenitor cells that can be identified by anatomic, antigenic, and biochemical profiles (Zhang et al., 2008). We thus hypothesize that the regulatory signals in fetal liver would be recapitulated in the cirrhotic liver.

To further validate if the same regulatory signals were clinically important and relevant in an *in vivo* stem cell regeneration model, we sought to analyze the genetic profile of microenvironment in which human fetal liver progenitor cells undergo proliferation, differentiation and maturation, *in vivo*.

Human adult cirrhotic liver vs. human fetal liver

We performed PCR gene array analysis for adult cirrhotic liver and compared the genes that were up regulated in both the fetal liver and the human adult liver versus human cirrhotic liver. We isolated RNA, and qRT-PCR array was performed comparing the fresh human cirrhotic liver samples with adult liver and fetal liver samples. We tested about 364 genes that were described to be involved in liver development by previous studies. The array data showed an interesting trend of cirrhotic liver gene expression was similar to the fetal liver week 22 during development. The genes that were significantly upregulated in the adult cirrhotic liver compared to adult liver sample were stem cell transcriptional factors (OCT4, SOX2, NODAL and NANOG), Hedgehog signaling, FGF8, BMP3, T (brachury) and HNF1b (**Figure 6.1**). Therefore this data supports our hypothesis of progenitor cell proliferation at week 22. This is evident as the upregulation of Stem cell markers.

5.2 Materials and methods:

5.2.1 qRT-PCR Array

Total RNA was isolated using Trizol reagent (Invitrogen, Austin, TX Catalog number: 15596-026), 10 week, 14week, 18 week, 22 week, human adult liver and cirrhotic liver. Isolated RNA was subjected to bioanalyser to check the integrity and used which was more than 8 in all our experiments.

5.2.2 Immunohistochemistry and Immunofluorescence

All the liver tissue was processed fresh. Sections for Immunofluorescence were fixed with 10% cold formalin. Sections were then blocked with appropriate serum 10% for 30 minutes. Primary antibodies against human antigens were applied to slides for overnight at 4 degrees and they include anti-albumin (ICN Pharmaceuticals, Aurora, OH), Fluorescent detection was by Alexa Fluor® 488 or Alexa Fluor® 594 conjugated secondary antibodies (Invitrogen, Carlsbad,CA). Nuclei were cross stained with 4',6-Diamidino-2-phenylindole (DAPI). Cell morphology was studied with Nikon Inverse phase contrast microscope and Immunofluorescence was captured by Olympus DP70 immunofluorescent microscope and Confocal Olympus inverted microscope. Positive cells was determined by manual counting of positive immunofluorescent cells to DAPI positive nucleus in 3 separate random fields (at 40X magnification objective) on the Olympus microscope.

5.2.3 Masson's trichrome staining.

The sections were stained with Masson's trichrome method which was used to distinguish and analyze for cavernous smooth muscle (stained in red) and collagen (blue) and expressed as the ratio of cavernous smooth muscle/collagen.

5.2.4Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is the abbreviation of enzyme-linked immunosorbent assay. It is a useful and powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine, sperm and culture supernatant. The basic principle

of an ELISA is to use an enzyme to detect the binding of antigen (Ag) antibody (Ab). The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Ags or Abs in a sample, depending on how the test is designed.

Human albumin concentrations from culture media were measured by the sandwich enzyme-linked immuno-sorbent assay as previously described. The capture antibody (goat anti-human antibody; 4 mg/mL), the detection antibody (peroxidase-conjugated goat immunoglobulin G fraction to human albumin; 3 mg/mL), and the purified human albumin standards were purchased from ICN Pharmaceuticals (Durham, NC).

5.2.5 Cirrhotic liver injury model

We have used the NSG mice for our cirrhotic live model for this study. N=3**.** Nod-scid gamma mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) with deleted B cells and T cells were obtained under MTA from the Jackson laboratory and bred in animal unit NUS that were used in transplantation experiment. Liver injury was induced by feeding the mice with Thioacetamide (TA) every day from 3 month to 10 months to induce liver cirrhosis.

Mouse histology was examined at 3 month, 4 month, 6 month and at 10 month and then the mice were sacrificed thereafter. Sirius red, trichrome staining and H&E stains were performed to understand the cellular architecture of changes in liver. H&E staining show a disorganized lobule formation. The changes in the

bridging of portal were analyzed with trichrome staining (**Figure 5.2, Figure 5.3 and Figure 5.4**).

5.2.6 Site of delivery to target organ

Fetal liver progenitor cells transplantation was performed via intrasplenic route. The intrasplenic route is technically easier and has been shown that almost all cells will migrate over to the liver by 24 hours. It has been previously demonstrated that the portal vein network traverses through the liver cords via the portal tract. The transplanted cells enter the hepatic parenchyma through this Protal vein.

5.2.7 Transplantation

Nod-scid gamma mice at 3 months were treated with 200ug/L of thioacetamide in drinking water each day till the sacrifice of the mice. Transplanted mice were administered with 2 million human fetal liver progenitor cells enriched in primary fetal liver cultures via the intrasplenic route. Control animals were given sham surgery but infused with equivalent 0.2 ml phosphate buffered saline intrasplenically. Mice were then sacrificed at 4^{th} month in order to determine if human progenitor cells persisted and followed up at month 10 to track the repopulation levels.

Results:

To realize the translational relevance, it is key to understand, what signals would the transplanted human fetal liver progenitor cells produce to integrate, differentiate and function like normal differentiated cells.

5.3 Localizing the engrafted human cells

5.3.1Human specific albumin to identify human hepatocytes

The transplanted cells were examined by immunofluorescence labeling with human albumin marker in mouse liver tissues. In mouse liver tissues, presence of human cell clusters was stained with albumin (**Figure 5.5**).

5.3.2 Functions of *in-vivo* human cells

Our aim is to demonstrate the ability of liver to restore its functions and identify the signals involved in the restoration of the liver cell populations.

5.3.4 Production of albumin into serum

Albumin production into the serum is a core function of hepatocytes and plays the role of carrier proteins for both nutrients and toxins as well as maintenance of oncotic pressure. The result from ELISA shows that the serum albumin levels were restored to normal levels in transplanted animals compared to control mice (**Figure 5.6A**).

5.3.5 Liver Function Test

Additionally we have shown that liver function on these rats improved. Blood was collected from the animals to study the liver functional test, namely periodic total bilirubin (TBIL) (**Figure 5.6B**).

5.5 Interpretation

SCID mice were used for these experiments as they were bred especially for human cell transplants. Human fetal liver cells are able to repopulate cirrhotic liver of mouse model of liver injury. They survive and differentiate into

functional liver cells, integrating with mouse hepatocytes. They express albumin, total bilurubin. In the transplanted cirrhotic animal model, we observed the upregulation of the SHH gene. Hence, we conclude that the SHH gene may have a potential role in driving the transplanted hepatocytes to differentiate and recover liver function.

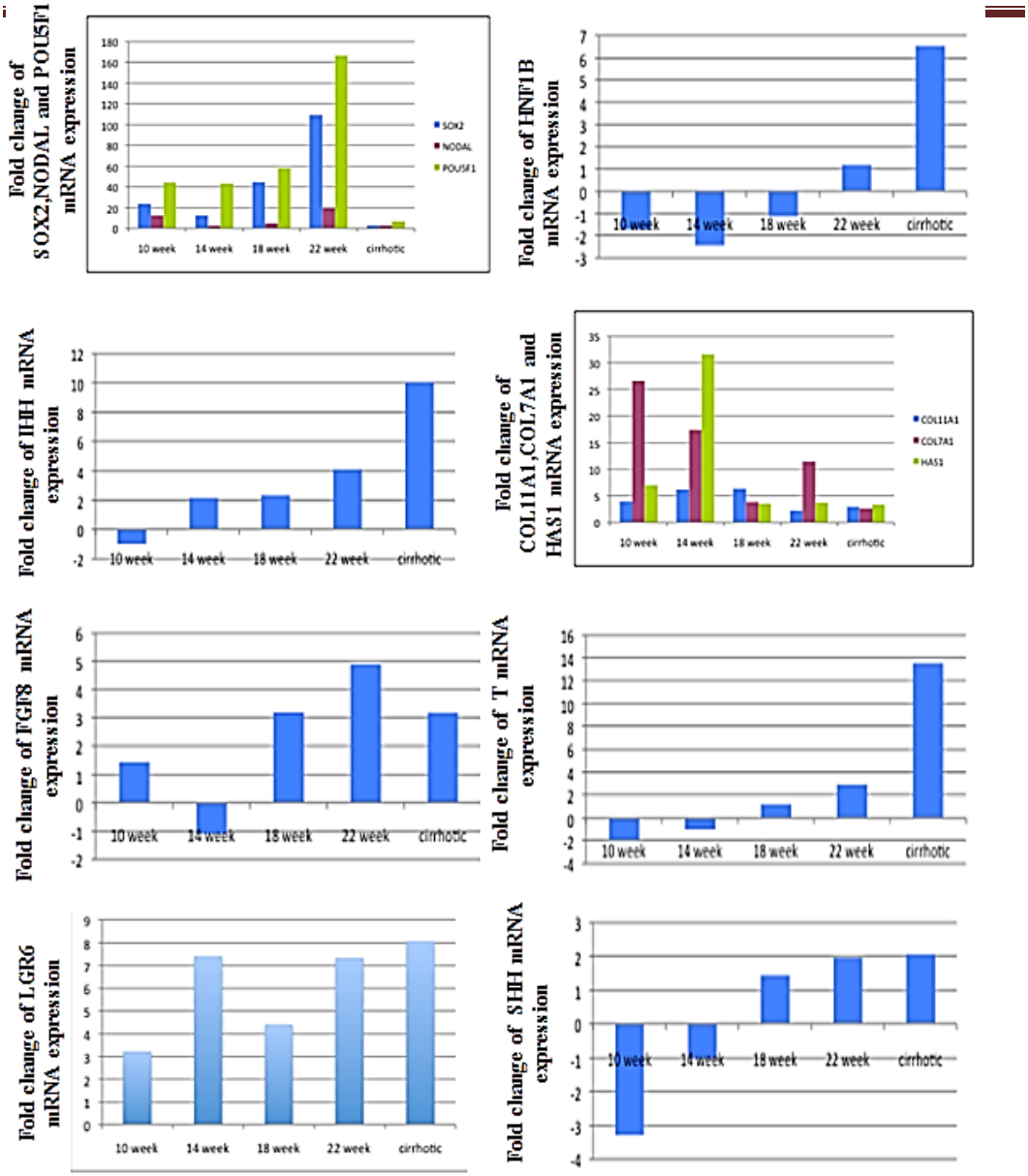


Figure 5.1 mRNA-expression levels of in developmental stages and cirrhotic liver were measured by real-time-PCR experiment.

cDNA was converted from first strand cDNA kit. The expression was normalized with the endogenous control of GAPDH. The comparison was made human normal adult liver control. The data shows the similarity of cirrhotic liver with week 22 of developmental stage. We notice the up regulation of stem cell transcriptional factors (OCT4, SOX2, NODAL and NANOG), IHH gene, FGF8, BMP3, T (brachury) and HNF1b. The interesting gene that we noticed was that the upregulation of SHH gene.

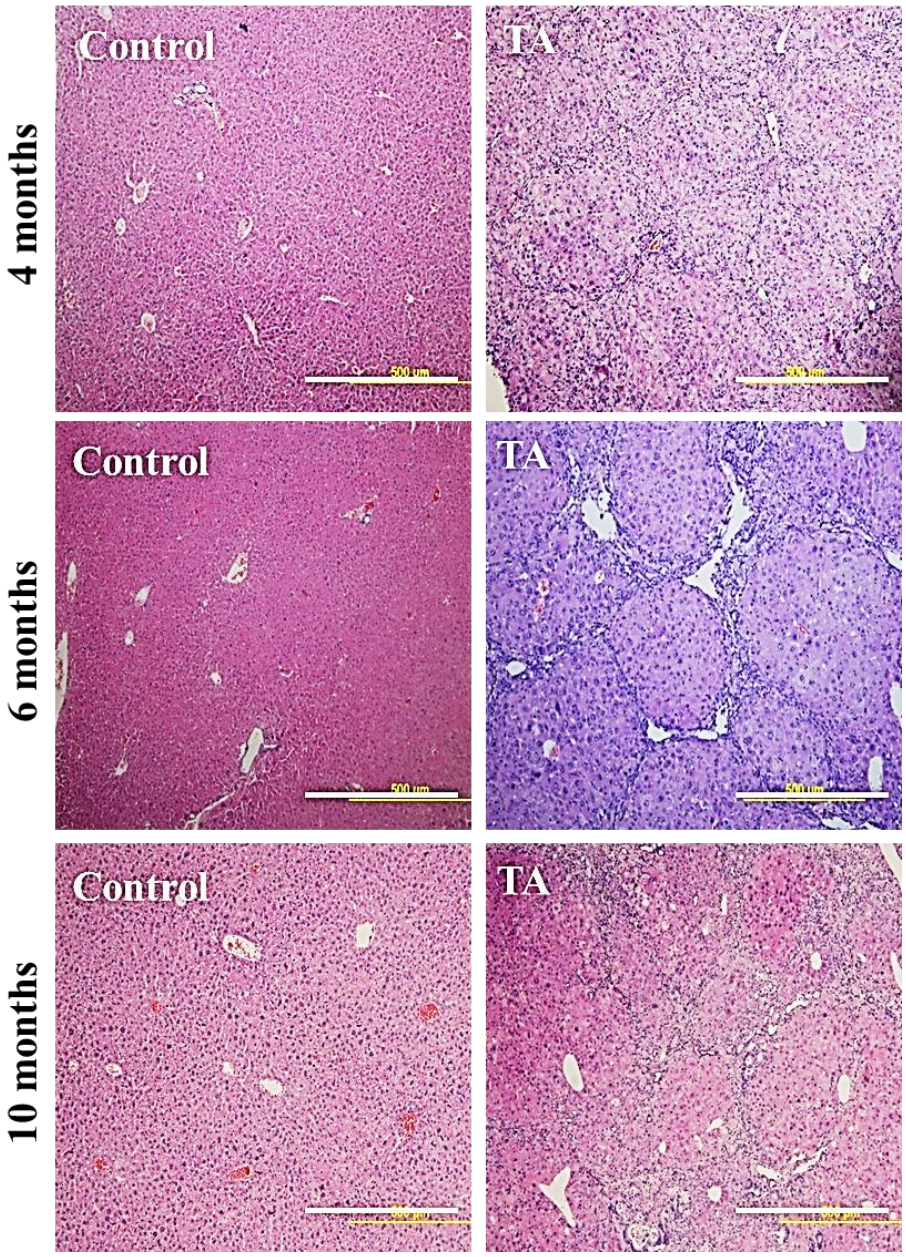


Figure 5.2 Liver cirrhosis model:

Photomicrographs of a section of the liver of group II rats showing: a Disorganized lobular pattern with the formation of pseudo lobules.

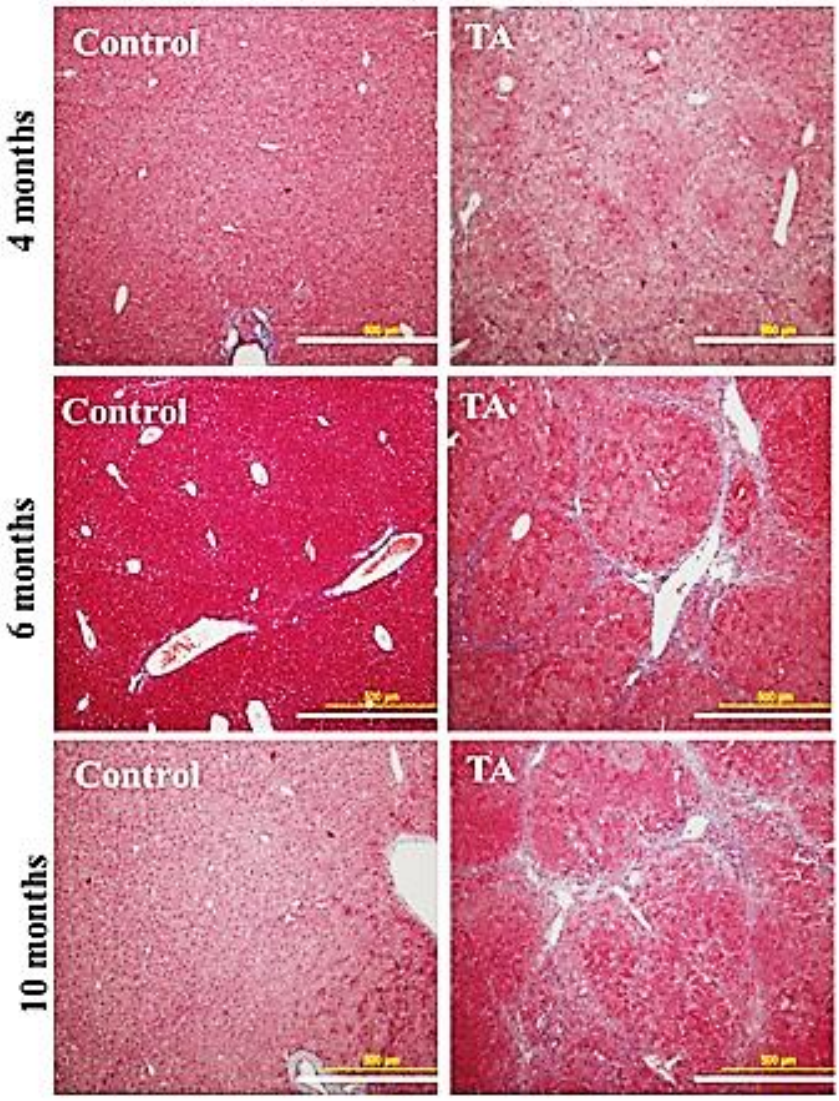


Figure 5.3 Sirius red staining showing the irreversible cirrhosis in animals.

The formalin/paraffin sections of liver tissue free of tumor nodules were stained with Sirius red. Each figure is a representative picture of three animals in each group at each time point. The collagen deposition was stained in red.

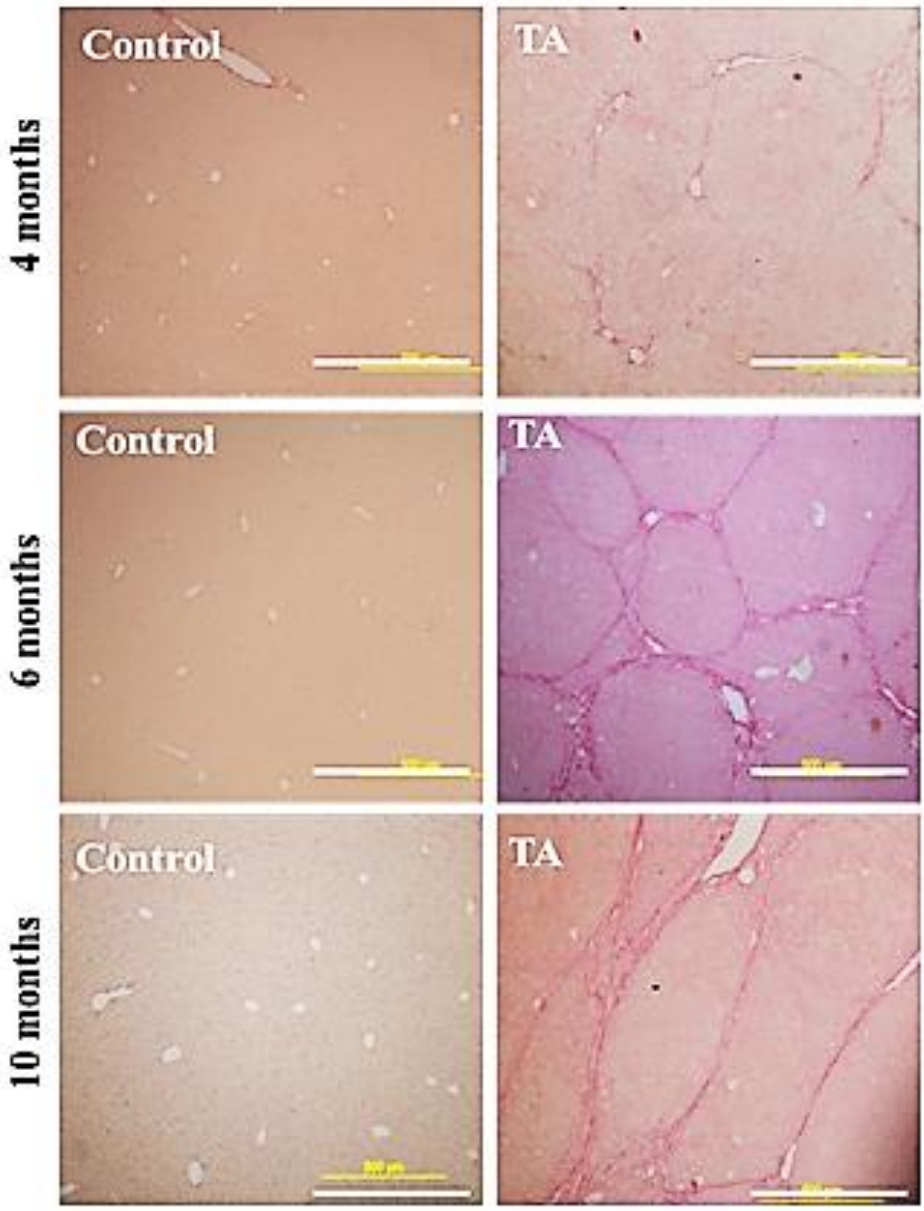


Figure 5.4 Masson trichrome stain.

Fibrous connective tissue septa are noted bridging the portal areas and extending into the lobules. Masson trichrome stain.

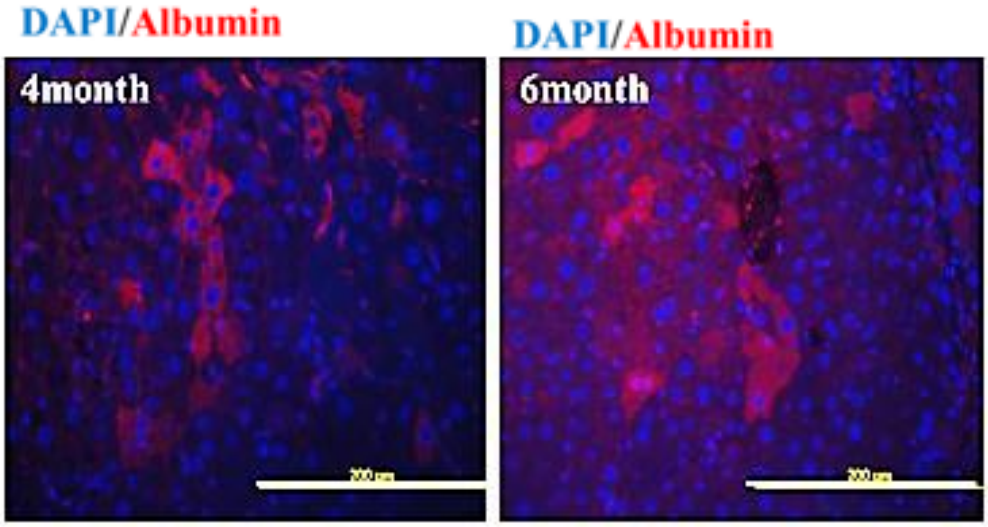


Figure 5.5 albumin in Immunofluorescence staining

Sections of transplanted mouse month 4 and month 6 post- transplant show human specific albumin in Immunofluorescence staining.

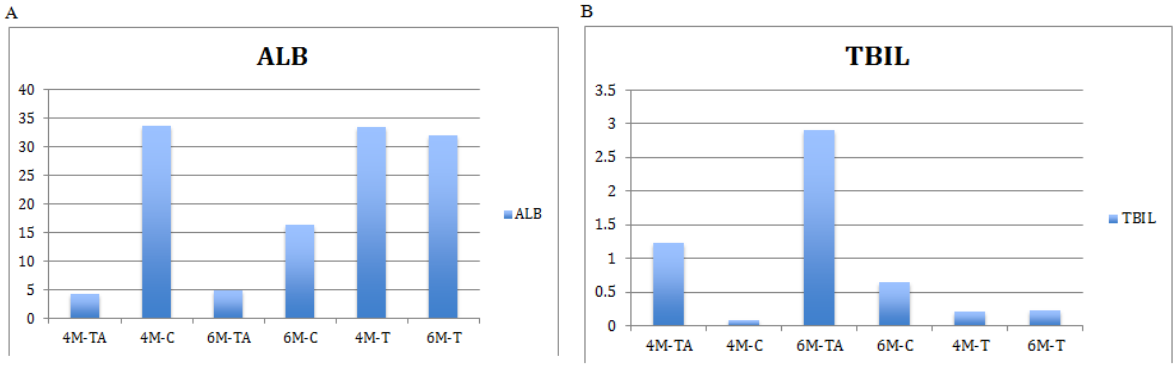


Figure 5.6 Liver function test

Liver function test shows the transplanted animals regain its functional properties. A. Albumin, B. Total Bilurubin. T - Transplanted, C- Control, M- Month, TA- Thioacetamide.4 and 6 represent the age of the mice.

Chapter 6

Discussion

In the past few decades, detailed analyses of liver development have been performed with various animal models tracing the formation of the liver from definitive endoderm to the mature liver. However, systematic analysis of the key genetic determinants that drive human fetal liver development has never been performed in such a systematic manner. Taking this into account, we demonstrate systematically, the molecular mechanism and its regulatory profile in developing human fetal liver. Insights into Human fetal liver development holds great promise in allowing us to apply the understanding of development of human hepatocytes to our efforts in expanding and growing hepatocytes ex vivo. This will enhance the culturing techniques by identifying the key signals such as signaling factors, growth factors and extracellular matrix niche. This is of critical importance as we embark on efforts to convert ES and IPS cells along their natural differentiation route and will hopefully allow us to break through the impasse to develop fully functional liver cells.

In our research we had unique privileged access to collect human fetal liver with full consent from the KK Women and Children's Hospital. We have used mid gestational weeks from 10 week till 23 week in this study. We determined the global gene expression profiles for each stage of the developmental process and applied state of the art gene interrogation to understand the genetic determinants controlling the development of the fetal liver.

Based on these data, we defined a subset of mRNAs whose detection can be used to determine phenotypic changes in specification, differentiation and

maturation of human fetal liver. In addition to being useful for phenotypic analyses, defining the gene expression profile for each stage will probably facilitate the identification of molecular pathways with undefined roles during human hepatocyte differentiation.

6.1 what are the regulatory phases of human fetal liver development?

We have observed that human fetal liver express five different phases in mid gestational period from week 10 till week 23. Comparison was made with embryonic Day 0 and contrasted with adult normal livers. Classical understanding of human fetal development has depended on immunohistochemistry and focus has been on the liver architecture. Similarly, we were able to observe what has been described of the ductal plate developing from a primitive structure at week 14 into a complete portal triad at week 22. Animal experiments have resulted in conventional concept of epithelial progenitor cells becoming hepatoblasts and progressively maturing into hepatocytes. Although a progenitor population has been described in fetal liver (Zhang et al), when and where this population arises from and the interactions with the hematopoietic phase is poorly understood.

Using immunophenotyping and transcript signature, 5 phases were discernible in fetal liver development:

(1) Stem cell specification phase till 10 weeks;

(2) Hepatoblast proliferation from 11 to 14 week gestation

(3) Hematopoiesis from 14 to 18 weeks

(4) Second wave of progenitor proliferation from 18 to 22 weeks

(5) Maturation from week 22 onwards to adult liver.

6.1.1 stem cell and hepatoblast specification phase

During 10 weeks we see the EpCAM cells appear surrounding the ductal plates which confirms the previous studies showing the ductal plates give rise to EpCAM+ stem cells. The EpCAM cells are believed to migrate outwards and differentiate into expanding hepatoblast that is seen around week 14. The EpCAM expression reduces in week 18 during the hematopoiesis phase and resurges in progenitor cell proliferation phase during week 22. CD44+ and CD133+ stem cells strongly express during week 10 consistent with a generic stem cell genotype. These stem cells genes including OCT4, SOX2, NODAL continue to be strongly upregulated from week 10 to week 14 and the expression is consistent with continued proliferation of the stem cells. At this stage, we also see upregulation of HNF1A genes indicating specification into the liver lineage. The surge in AFP and albumin at week 10-14 is consistent with observation of clusters of AFP + cells emanating from the ductal plate.

6.1.2 Hematopoietic phase

Human fetal liver acts as a primary hematopoietic organ during organogenesis. Hematopoietic cells reside in liver during the prenatal stage and move to the bone marrow during postnatal stage. Fetal liver Immunofluorescence CD45 staining (markers for hematopoietic origin) was performed and it was noted that

hematopoietic cells surge in numbers during the 18 weeks of fetal liver development around the ductal plates and portal tracts and these population of cells dwindle in expression from week 20 onwards. The microenvironment in the liver changes where OSM gene secreted from hematopoietic cells induce the hepatic transcriptional factors such as HNF and HGF growth factor for hepatocyte differentiation and proliferation. We found that the hematopoietic stem cell activating factor XSIT up regulated during week 18, however XSIT repressor gene TISX highly up regulate to suppress the transcriptional activity. This change in microenvironment is suitable for hepatic proliferation where the hematopoietic stem cells leave the liver and reside in bone marrow.

6.1.3 Maturation and Expansion

After the exodus of hematopoietic stem cells, hepatocytic maturation factors such as OSM, HGF and FGFR1 signaling is up regulated together with JAK-STAT pathway in week 22. JAK-STAT pathway is important in maturation and differentiation during embryogenesis. During this phase we see the upregulation of epithelial growth factor EGF that is an important factor in epithelial cell maintenance.

6.2 Transcriptional regulation of Human fetal liver development

In addition to the genetic variation that are regulated in different stages we have determined the key genes that are upregulated during different stages of development. The key transcription factors that showed greater than 2-fold up regulation in 10-14 weeks were GATA4, HNF3 and HNF4a, consistent with

specification and these had a sudden up regulation from 22 weeks onwards with all the important genes like SHH, DLL, DLX, PATCH, NODAL involved in Hedge hog pathway were upregulated in week 22. At 18-21 weeks, EGF, FGF4, BMP3 and extracellular matrix genes like COL12A1, COL7A1, ITGA3, LAMA3 were upregulated >2 fold compared to 14 and 22 weeks of fetal liver as well as normal liver. At 22 weeks the stem cell genes like POU5F1, SOX2, CD44, NANOG are significantly upregulated compared to 10 weeks in 22 samples.

6.3. In vitro evidence of differentiation and maturation of fetal liver

In addition to providing proof-of-concept we have demonstrated the in vitro application of growth factors (FGFr and CTGF) Extracellular matrix (collagen, lamanin and hyualuranic acid) that can be used as an efficient tool to probe human fetal cell fate. We exploited the use of growth factors CTGF and ECM proteins in controlling the onset of human hepatocyte differentiation and expansion.

6.3.1 Optimizing cultures to ECM and growth factors

Recently the focus on controlling the microenvironment of the in vitro plating has provided remarkable progress in cell culture techniques to stimulate proliferation and maturation of cell functions. However, the drawbacks of using current techniques, is difficult to maintain long-term expression of liver-specific functions. Previous studies are shown to demonstrate the reconstruction of culture systems to more closely mimic the native in vivo microenvironment using ECM components. These studies succeeded in promoting expression of liver-

specific functions of primary rat hepatocytes. It is therefore effective to mimic the in vivo microenvironment for ensuring expression of organ-specific functions and stem cell differentiation.

We have used collagen, lamanin and Hyauronic acid as a basement support combination to promote human growth and differentiation. Human Fetal liver Cells were collected freshly and dissociated for culture. Our results have shown successful maintenance of human fetal liver cells using a combination of collagen and lamanin but lost its phenotypic characteristics when cultured in hyaluronic acid. Cell-cell contact appears to be critical for maintenance of these cells. The limitation of using specific ECM isoforms like collagen and lamanin are the availability of these products in market. The ability to create the exact isoform of the collagen molecules and lamanin will give better microenvironment required for the liver cell development.

Modern molecular techniques have showed that members of the fibroblast growth factor family FGF1 and FGF2 in inducing the onset of albumin expression, a characteristic marker of hepatic cell fate in explants of mouse anterior endoderm. FGF growth factors have shown to mediate specification of hepatic cell fate in a concentration dependent manner in cell culture. FGF is also appearing to be controlled by endoderm relative to the heart, which is the major source of hepatogenesis FGF during liver development. Several FGFs including *Fgf1*, *Fgf2*, *Fgf8*, and *Fgf10* are expressed in the mesoderm during hepatogenesis, and knockout studies in mice suggest that these factors play an important role in controlling the liver development. The requirement for FGF

signaling in controlling the onset of liver development is evolutionarily conserved, with FGFs displaying hepatogenic properties in *Xenopus*, chick, and *Zebrafish* embryos. We show in our study the importance of the FGF receptors FGFR1 and FGFR2. We showed that the blocking of these receptors with the specific antibodies has control in expansion and proliferation of the fetal liver cells. The FGFR blocking has increased the expansion of the liver cells in culture from more than a month without losing their phenotypic characteristics.

6.4. Potential of therapeutic effect of cell transplantation

To document principle of proof of fetal liver cells in being to have a therapeutic effect, we created animal model using thioacetamide (TA) diet to create progressive liver injury. Fetal liver cells were transplanted and showed improvement in liver function and reversal of fibrosis. While this served as principle of proof that cell transplantation may benefit patients with liver cirrhosis. The current challenge is to find appropriate sources of cells and yet being able to differentiate them into functional hepatocytes. Using the somatic progenitor cell as an example, we were able to precisely profile the cells and map the cells in the progeny pathway of liver differentiation. This allows a systematic approach for specific manipulation to move these cells along the differentiation pathway.

6.5 Limitations

As we have discussed the important findings of our study, we have few points to elaborate the limitations that we think should be addressed.

1. Though we state an in depth analysis of the mid gestational period of liver development, we observed the maturation of the liver is not yet complete at week 22. In real life, liver maturation continues in neonate up to 12 years old before the hepatocytes become fully mature. The clinical material from these time points are lacking due to ethical concerns and practical issues. This period of development would be very crucial in understanding the important maturational signal and extracellular niche that maintain the functional hepatocyte as well drive the maturation.

2. Similarly human data from ES cells till week 10 are not easily studied in vivo and what is understood is extrapolated from animal models.

3. Rather than a simple system of best or fixed combination of extracellular matrix to support ex-vivo culture, the in vivo micro environmental niche is likely complex where the extracellular matrix composition is dynamic and changes temporally as well as spatially in different parts of the fetal liver. Whole tissue RNA analysis does not allow that degree of resolution. Even the recapitulation of the dynamic ECM environmental niche will be difficult to replicate in ex-vivo culture.

4. When we use whole fetal tissue samples as the source there are chances that we are focusing on the mixed cell population in the fetal liver development, where the gene expressional change in the cell (hepatocytes) that we are interested in would be diluted because of the relative expression change as a whole.

5. In our genetic array study we have used 2-fold change as the threshold for the upregulation of the genes. This is based on conventional and practical assumptions that some of the important master regulator transcriptional factors are highly expressed in fold change more than 2 fold. There is thus, a real chance of missing out the important factors that may be critically and finely regulated but below the 2-fold change threshold.

6. Microarray analysis typically suffers from dangers of reductionist approach in inevitable complexities of biological systems..One example is the FGF signaling. We have shown that the FGF signaling is upregulated in the development but yet in vitro, the process is complex with growth factors, receptors upregulation, redundancy etc. Such complex systems cannot be unraveled by genetic studies. Nevertheless, the hope is that there will be several key regulators that can be identified and targeted for intervention.

6.6 Future Directions

1. We have noticed the sudden upregulation of EpCAM positive populations of cells in week 22. We have shown them to be the mesenchymal epithelial positive populations. The next questions we ask is that 1. What are these cells? And 2. What is the source of these cells? In order to identify the source of the cells we can use Cre-lox study for cell fate mapping in the animal model. Use 14 week, 18 week and 22 week non-parenchyma cells to identify the nature and source of the cells that are undergoing mesenchymal epithelial transition.

2. In our in vitro chapter we have described about the SHH gene is important in differentiation of the hepatocytes. This data is currently not congruent with current literature. Further validation is needed to see whether the cells with and without SHH produce the hepatic cells. We need to fate map the cells with cre-lox SHH gene and transplant them in vitro to study the differentiation.

3. The physiological roles of liver progenitor cells at 18-22 weeks continue to be an interesting question. Do they contribute much to the adult hepatocyte fraction? Do they play a pure reserve role in repair?. Cre-lox MET tracing of fetal liver progenitor cells in animal fetal models will be useful in defining this.

4. From our microarray data we have noticed a broad spectrum of genes that upregulated in the top 600 list and are yet to be understood the function of

it in liver. This widens the gap in knowledge about the new genes that can be studied in liver development. Similarly the new avenue of miRNA profiling in liver development is subject of open question. Here we propose to identify the miRNA profiling of the human fetal liver development that can be used in translational medicine in the near future.

6.7 Conclusion

Human fetal liver from 11 weeks to 24 weeks gestation were obtained and analysed with IHC, microarray, next generation sequencing and qRT PCR for growth factor, extracellular matrix and liver specification transcriptional factors. Comparison was made with 10 week fetal liver and normal adult liver. Using immunophenotyping and transcript signature, 5 phases were distinguished and categorized in fetal liver development. Fetal liver progenitor cell undergoes proliferation at 10 to 14 week gestation, from 14 to 18 weeks differentiation of fetal hepatic lineage - the hepatoblast form, in week 18 there are signs of hematopoietic lineage expression from liver and from week 20 onwards there is a sudden proliferation of EpCAM and Vimentin positive cells, which is seems to be a mesenchymal lineage.

EPCAM/CD44+ progenitor cells appear at 10 weeks and undergo surge in proliferation at 18-20 weeks of gestation before dwindling in frequency. The expression pattern of EPCAM corroborate that there are distinct phases such as progenitor cell population surge at 10 weeks, hepatoblast proliferation from 10 to 14 weeks, hematopoietic phase at 15-18 weeks and reactivation to 22 weeks. This suggests that epithelial cell proliferation appears to take a backstage during the hematopoietic phase but undergo a second wave of progenitor cell proliferation with resurgence with termination of the hematopoietic phase. This was accompanied by increase in albumin and CYP450 gene expression compared to the 10 week fetal liver validating the maturation of fetal liver at this stage.

Week 10-11 Specification phase - genes at this stage were mostly embryonic stem cell factors and would be useful for in vitro IPSC manipulation. Week 14-15: Expansion / differentiation phase - Genes at this stage were extracellular matrix proteins Collagen, Laminin and Hyaluronic acid. Week 17-19: Hematopoiesis and Proliferation Week 20-24: Maturation, EMT phase - genes that were upregulated at this stage were FGF, FGFR and CTGF. Adult Liver: functional. Specifically, week 11 to 14 would represent the phase of hepatoblast playing the role of a transit-amplifying cell in expansion and differentiation into hepatic lineage. Similarly, week 19 to 22 would represent the phase of progenitor cell proliferation by mesenchymal epithelial transition. Week 23 to adult would represent the phase of hepatocyte maturation.

We have also identified key genes in each phase and are classified under transcriptional factors (GATA4, FOXA2 and CEBPa), growth factors (FGFR and CTGF) and extracellular matrix (COL12A1, LAMA3, Fibronectin and Hyurolic Acid). The transcriptional factors were identified as key signaling factors that are important in direct reprogramming of fibroblast to hepatocytes. To test if SHH was driving the differentiation, SHH supplementation in fetal liver cultures showed differentiation with maintenance of immunophenotyping. Inhibiting the SHH resulted in proliferation and expansion of liver cells. These liver cells were transplanted into SCID mice treated with thioacetamide and the degree of repopulation was analyzed and correlated with growth factors and extracellular growth matrix. In vivo transplantation of these cells in mice livers showed positive correlation of human cells engraftment. However using this knowledge

in understanding their key regulation of liver development will help in expanding progenitor cells for regenerative purposes. With further validation these kind of systematic analysis will be a useful in identifying the key genes that would be essential in regenerative medicine.

Liver Specific		Growth Factors		Extra cellular Matrix	
ALB	APOF	ABCG2	KAT2A	ADAMTS1	LAMB1
CDH1	T	ACTC1	GDF2	ADAMTS13	LAMB3
CYP2C9	CEBPA	ADAR	GDF3	ADAMTS8	LAMC1
GSK3A	CEBPB	ACAN	GJA1	CD44	MMP1
LTA	CYP2B6	ALDH1A1	GJB1	CDH1	MMP10
SERPINA1	CYP3A4	ALDH2	GJB2	CNTN1	MMP11
ABCB11	CYP7A1	ALPI	HDAC2	COL11A1	MMP12
CEBPA	EGF	APC	HSPA9	COL12A1	MMP13
CYP2D6	EOMES	ASCL2	IGF1	COL14A1	MMP14
MMEX	FGFR2	AXIN1	PDX1	COL15A1	MMP15
MIXL1	FOXA1	BGLAP	ISL1	COL16A1	MMP16
SOX2	G6PC	BMP1	JAG1	COL1A1	MMP2
ADH1C	GATA4	BMP2	KRT15	COL4A2	MMP3
CPS1	GATA6	BMP3	MME	COL5A1	MMP7
CYP2E1	GLI1	BTRC	MSX1	COL6A1	MMP8
KLF4	GLI2	CCNA2	MYC	COL6A2	MMP9
NANOG	GLI3	CCND1	MYOD1	COL7A1	NCAM1
SOX7	HGF	CCND2	KAT8	COL8A1	PECAM1
AFP	HNF1A	CCNE1	KAT7	VCAN	SELE
RY2	HNF1B	CD3D	NCAM1	CTGF	SELL
DLK1	HNF4A	CD4	NEUROG2	CTNNA1	SELP
KLHL1	IHH	CD44	NOTCH1	CTNNB1	SGCE
NCAM1	KRT7	CD8A	NOTCH2	CTNND1	SPARC
TSPAN7	KRT18	CD8B	NUMB	CTNND2	SPG7
ANXA10	ONECUT2	CDK1	SIGMAR1	ECM1	SPP1
CTNNB1	OSM	CDC42	PARD6A	FN1	TGFBI
EPCAM	PCK2	CDH1	PPARD	HAS1	THBS1
KRT19	PDGFRB	CDH2	PPARG	ICAM1	THBS2

NODAL	PROM1	COL1A1	RB1	ITGA1	THBS3
GADPH	PROX1	COL2A1	S100B	ITGA2	TIMP1
BMP4	PTCH1	COL9A1	SOX1	ITGA3	TIMP2
CXCR4	PTCH2	CTNNA1	SOX2	ITGA4	TIMP3
FGF8	SHH	CXCL12	T	ITGA5	CLEC3B
KRT7	SMAD2	DHH	TERT	ITGA6	TNC
OTC	SMAD3	DLL1	TUBB3	ITGA7	VCAM1
GDC	SMAD4	DLL3	WNT1	ITGA8	VTN
CD24	SMAD5	DTX1	B2M	ITGAL	B2M
CYP1A2	SMO	DTX2	HPRT1	ITGAM	HPRT1
GJB1	SOX17	DVL1	RPL13A	ITGAV	RPL13A
LGR5	TAT	EP300	GAPDH	ITGB1	GAPDH
PCK1	TGFB2	FGF1	ACTB	ITGB2	ACTB
RTC	TGFB3	FGF2	HGDC	ITGB3	HGDC
CD44	THY1	FGF3	RTC	ITGB4	RTC
CYP2C19	TTR	FGF4	RTC	ITGB5	RTC
GSC	GAPDH	FGFR1	RTC	KAL1	RTC
LGR6	HGDC	FOXA2	PPC	LAMA1	PPC
POU5F1	RTC	FRAT1	PPC	LAMA2	PPC
PPC	PPC	FZD1	PPC	LAMA3	PPC

References

Agarwal S, Holton KL, Lanza R (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. Stem Cells 26:1117-1127.

Ang SL, Wierda A, Wong D, Stevens KA, Cascio S, Rossant J, Zaret KS (1993) The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development 119:1301-1315.

Asahina K, Zhou B, Pu WT, Tsukamoto H (2011) Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. Hepatology 53:983-995.

Bellamy CO, DiMartini AM, Ruppert K, Jain A, Dodson F, Torbenson M, Starzl TE, Fung JJ, Demetris AJ (2001) Liver transplantation for alcoholic cirrhosis: long term follow-up and impact of disease recurrence. Transplantation 72:619-626.

Bhushan A, Itoh N, Kato S, Thiery JP, Czernichow P, Bellusci S, Scharfmann R (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128:5109-5117.

Borrello S, De Leo ME, Galeotti T (1992) Transcriptional regulation of MnSOD by manganese in the liver of manganese-deficient mice and during rat development. Biochem Int 28:595-601.

Bossard P, Zaret KS (1998) GATA transcription factors as potentiators of gut endoderm differentiation. Development 125:4909-4917.

Calmont A, Wandzioch E, Tremblay KD, Minowada G, Kaestner KH, Martin GR, Zaret KS (2006) An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. Developmental cell 11:339-348.

Cascio S, Zaret KS (1991) Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. Development 113:217-225.

Chagraoui J, Lepage-Noll A, Anjo A, Uzan G, Charbord P (2003) Fetal liver stroma consists of cells in epithelial-to-mesenchymal transition. Blood 101:2973-2982.

Chen JK, Taipale J, Cooper MK, Beachy PA (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev 16:2743-2748.

Christensen JL, Wright DE, Wagers AJ, Weissman IL (2004) Circulation and chemotaxis of fetal hematopoietic stem cells. PLoS biology 2:E75.

Cleaver O, Melton DA (2003) Endothelial signaling during development. Nature medicine 9:661-668.

Cowling T, Jennings LW, Goldstein RM, Sanchez EQ, Chinnakotla S, Klintmalm GB, Levy MF (2004) Societal reintegration after liver transplantation: findings in alcohol-related and non-alcohol-related transplant recipients. Annals of surgery 239:93-98.

D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534-1541.

Dan YY, Riehle KJ, Lazaro C, Teoh N, Haque J, Campbell JS, Fausto N (2006) Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. Proceedings of the National Academy of Sciences of the United States of America 103:9912-9917.

Dan YY, Yeoh GC (2008) Liver stem cells: a scientific and clinical perspective. Journal of gastroenterology and hepatology 23:687-698.

de Boer CJ, van Krieken JH, Janssen-van Rhijn CM, Litvinov SV (1999) Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. The Journal of pathology 188:201-206.

Delgado JP, Parouchev A, Allain JE, Pennarun G, Gauthier LR, Dutrillaux AM, Dutrillaux B, Di Santo J, Capron F, Boussin FD, Weber A (2005) Long-term controlled immortalization of a primate hepatic progenitor cell line after Simian virus 40 T-Antigen gene transfer. Oncogene 24:541-551.

Deutsch G, Jung J, Zheng M, Lora J, Zaret KS (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128:871-881.

Douarin NM (1975) An experimental analysis of liver development. Medical biology 53:427-455.

Duncan SA (2000) Transcriptional regulation of liver development. Dev Dyn 219:131-142.

Duncan SA (2003a) Mechanisms controlling early development of the liver. Mech Dev 120:19-33.

Duncan SA (2003b) Mechanisms controlling early development of the liver. Mechanisms of development 120:19-33.

Duncan SA, Nagy A, Chan W (1997) Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of Hnf-4(-/-) embryos. Development 124:279-287.

Enzan H, Himeno H, Hiroi M, Kiyoku H, Saibara T, Onishi S (1997) Development of hepatic sinusoidal structure with special reference to the Ito cells. Microscopy research and technique 39:336-349.

Fassler R, Meyer M (1995) Consequences of lack of beta 1 integrin gene expression in mice. Genes & development 9:1896-1908.

Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology (Baltimore, Md 39:1477-1487.

Fernando RI, Litzinger M, Trono P, Hamilton DH, Schlom J, Palena C (2010) The T-box transcription factor Brachyury promotes epithelial-mesenchymal transition in human tumor cells. The Journal of clinical investigation 120:533-544.

Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC (1998) Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. The New England journal of medicine 338:1422-1426.

Fukuda-Taira S (1981) Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm. Journal of embryology and experimental morphology 63:111-125.

Gallacher L, Murdoch B, Wu D, Karanu F, Fellows F, Bhatia M (2000) Identification of novel circulating human embryonic blood stem cells. Blood 96:1740-1747.

Govaere O, Komuta M, Berkers J, Spee B, Janssen C, de Luca F, Katoonizadeh A, Wouters J, van Kempen LC, Durnez A, Verslype C, De Kock J, Rogiers V, van Grunsven LA, Topal B, Pirenne J, Vankelecom H, Nevens F, van den Oord J, Pinzani M, Roskams T (2014)

Keratin 19: a key role player in the invasion of human hepatocellular carcinomas. Gut 63:674-685.

Greenow K, Clarke AR (2012) Controlling the stem cell compartment and regeneration in vivo: the role of pluripotency pathways. Physiological reviews 92:75-99.

Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS (1996) Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes & development 10:1670-1682.

Hentsch B, Lyons I, Li R, Hartley L, Lints TJ, Adams JM, Harvey RP (1996) Hlx homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. Genes & development 10:70-79.

Jones EA, Clement-Jones M, James OF, Wilson DI (2001) Differences between human and mouse alpha-fetoprotein expression during early development. Journal of anatomy 198:555-559.

Jung J, Zheng M, Goldfarb M, Zaret KS (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284:1998-2003.

Kaestner KH, Katz J, Liu Y, Drucker DJ, Schutz G (1999) Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. Genes & development 13:495-504.

Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. The EMBO journal 18:2127-2136.

Kamiya A, Kinoshita T, Miyajima A (2001) Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. FEBS letters 492:90-94.

Keng VW, Fujimori KE, Myint Z, Tamamaki N, Nojyo Y, Noguchi T (1998) Expression of Hex mRNA in early murine postimplantation embryo development. FEBS letters 426:183-186.

Kiel MJ, Morrison SJ (2008) Uncertainty in the niches that maintain haematopoietic stem cells. Nature reviews Immunology 8:290-301.

Kim SK, and D. A. Melton. (1998) Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. Proc Natl Acad Sci USA 95:13036-13041.

Kinoshita T, Miyajima A (2002) Cytokine regulation of liver development. Biochimica et biophysica acta 1592:303-312.

Kmiec Z (2001) Cooperation of liver cells in health and disease. Advances in anatomy, embryology, and cell biology 161:III-XIII, 1-151.

Kubota H, Reid LM (2000) Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proceedings of the National Academy of Sciences of the United States of America 97:12132-12137.

Kumashiro Y, Teramoto K, Shimizu-Saito K, Asahina K, Teraoka H, Arii S (2005) Isolation of hepatocyte-like cells from mouse embryoid body cells. Transplant Proc 37:299-300.

Lai E, Prezioso VR, Tao WF, Chen WS, Darnell JE, Jr. (1991) Hepatocyte nuclear factor 3 alpha belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork head. Genes & development 5:416-427.

Lamouille S, Xu J, Derynck R (2014) Molecular mechanisms of epithelial-mesenchymal transition. Nature reviews Molecular cell biology 15:178-196.

Lavine KJ, Yu K, White AC, Zhang X, Smith C, Partanen J, Ornitz DM (2005) Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. Developmental cell 8:85-95.

Lazaro CA, Croager EJ, Mitchell C, Campbell JS, Yu C, Foraker J, Rhim JA, Yeoh GC, Fausto N (2003) Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology 38:1095-1106.

Lee JS, Ward WO, Knapp G, Ren H, Vallanat B, Abbott B, Ho K, Karp SJ, Corton JC (2012) Transcriptional ontogeny of the developing liver. BMC genomics 13:33.

Leffert H, Moran T, Sell S, Skelly H, Ibsen K, Mueller M, Arias I (1978) Growth state-dependent phenotypes of adult hepatocytes in primary monolayer culture. Proceedings of the National Academy of Sciences of the United States of America 75:1834-1838.

Lemaigre F, Zaret KS (2004) Liver development update: new embryo models, cell lineage control, and morphogenesis. Current opinion in genetics & development 14:582-590.

Lemaigre FP (2003) Development of the biliary tract. Mechanisms of development 120:81-87.

Lemaigre FP (2009) Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 137:62-79.

Li J, Ning G, Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. Genes & development 14:464-474.

Lin S, Lin Y, Nery JR, Urich MA, Breschi A, Davis CA, Dobin A, Zaleski C, Beer MA, Chapman WC, Gingeras TR, Ecker JR, Snyder MP (2014) Comparison of the transcriptional landscapes between human and mouse tissues. Proceedings of the National Academy of Sciences of the United States of America 111:17224-17229.

Liu Y (2004) Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. Journal of the American Society of Nephrology : JASN 15:1-12.

Medlock ES, Haar JL (1983) The liver hemopoietic environment: I. Developing hepatocytes and their role in fetal hemopoiesis. The Anatomical record 207:31-41.

Michalopoulos GK, Bowen WC, Mule K, Stolz DB (2001) Histological organization in hepatocyte organoid cultures. The American journal of pathology 159:1877-1887.

Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276:60-66.

Monaghan AP, Kaestner KH, Grau E, Schutz G (1993) Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. Development 119:567-578.

Oertel M, Menthena A, Chen YQ, Teisner B, Jensen CH, Shafritz DA (2008) Purification of fetal liver stem/progenitor cells containing all the repopulation potential for normal adult rat liver. Gastroenterology 134:823-832.

Ohno N, Izawa A, Hattori M, Kageyama R, Sudo T (2001) dlk inhibits stem cell factor-induced colony formation of murine hematopoietic progenitors: Hes-1-independent effect. Stem cells 19:71-79.

Oliver G, Sosa-Pineda B, Geisendorf S, Spana EP, Doe CQ, Gruss P (1993) Prox 1, a prospero-related homeobox gene expressed during mouse development. Mechanisms of development 44:3-16.

Omenetti A, Choi S, Michelotti G, Diehl AM (2011) Hedgehog signaling in the liver. Journal of hepatology 54:366-373.

Omenetti A, Syn WK, Jung Y, Francis H, Porrello A, Witek RP, Choi SS, Yang L, Mayo MJ, Gershwin ME, Alpini G, Diehl AM (2009) Repair-related activation of hedgehog signaling promotes cholangiocyte chemokine production. Hepatology 50:518-527.

Rao MS, Khan AA, Parveen N, Habeeb MA, Habibullah CM, Pande G (2008) Characterization of hepatic progenitors from human fetal liver during second trimester. World journal of gastroenterology : WJG 14:5730-5737.

Rogler LE (1997) Selective bipotential differentiation of mouse embryonic hepatoblasts in vitro. The American journal of pathology 150:591-602.

Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes & development 15:1998-2009.

Rozga J (2006) Liver support technology--an update. Xenotransplantation 13:380-389.

Sasaki H, Hogan BL (1993) Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development 118:47-59.

Savarese F, Flahndorfer K, Jaenisch R, Busslinger M, Wutz A (2006) Hematopoietic precursor cells transiently reestablish permissiveness for X inactivation. Molecular and cellular biology 26:7167-7177.

Scadden DT (2006) The stem-cell niche as an entity of action. Nature 441:1075-1079.

Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373:699-702.

Schwartz M (2014) Perspective: incision revision. Nature 516:S17.

Shivtiel S, Kollet O, Lapid K, Schajnovitz A, Goichberg P, Kalinkovich A, Shezen E, Tesio M, Netzer N, Petit I, Sharir A, Lapidot T (2008) CD45 regulates retention, motility, and numbers of hematopoietic progenitors, and affects osteoclast remodeling of metaphyseal trabecules. The Journal of experimental medicine 205:2381-2395.

Sicklick JK, Li YX, Choi SS, Qi Y, Chen W, Bustamante M, Huang J, Zdanowicz M, Camp T, Torbenson MS, Rojkind M, Diehl AM (2005) Role for hedgehog signaling in hepatic stellate cell activation and viability. Lab Invest 85:1368-1380.

Stark KL, McMahon JA, McMahon AP (1991) FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. Development 113:641-651.

Sugi Y, Lough J (1995) Activin-A and FGF-2 mimic the inductive effects of anterior endoderm on terminal cardiac myogenesis in vitro. Developmental biology 168:567-574.

Sussman NL, Gislason GT, Conlin CA, Kelly JH (1994) The Hepatix extracorporeal liver assist device: initial clinical experience. Artificial organs 18:390-396.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Tanaka M, Okabe M, Suzuki K, Kamiya Y, Tsukahara Y, Saito S, Miyajima A (2009) Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. Mechanisms of development 126:665-676.

Timchenko NA, Harris TE, Wilde M, Bilyeu TA, Burgess-Beusse BL, Finegold MJ, Darlington GJ (1997) CCAAT/enhancer binding protein alpha regulates p21 protein and hepatocyte proliferation in newborn mice. Molecular and cellular biology 17:7353-7361.

Tremblay KD, Zaret KS (2005) Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. Developmental biology 280:87-99.

Weinstein M, Monga SP, Liu Y, Brodie SG, Tang Y, Li C, Mishra L, Deng CX (2001) Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. Molecular and cellular biology 21:5122-5131.

Yu Y, Ping J, Chen H, Jiao L, Zheng S, Han ZG, Hao P, Huang J (2010) A comparative analysis of liver transcriptome suggests divergent liver function among human, mouse and rat. Genomics 96:281-289.

Yu Y, Zhang C, Zhou G, Wu S, Qu X, Wei H, Xing G, Dong C, Zhai Y, Wan J, Ouyang S, Li L, Zhang S, Zhou K, Zhang Y, Wu C, He F (2001) Gene expression profiling in human fetal liver and identification of tissue- and developmental-stage-specific genes through compiled expression profiles and efficient cloning of full-length cDNAs. Genome research 11:1392-1403.

Zaret K (1999) Developmental competence of the gut endoderm: genetic potentiation by GATA and HNF3/fork head proteins. Developmental biology 209:1-10.

Zaret KS (2002) Regulatory phases of early liver development: paradigms of organogenesis. Nature reviews Genetics 3:499-512.

Zaret KS (2008) Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nature reviews Genetics 9:329-340.

Zarling JM, Shoyab M, Marquardt H, Hanson MB, Lioubin MN, Todaro GJ (1986) Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. Proceedings of the National Academy of Sciences of the United States of America 83:9739-9743.

Zhang L, Theise N, Chua M, Reid LM (2008) The stem cell niche of human livers: symmetry between development and regeneration. Hepatology 48:1598-1607.