

**GRAFTING TECHNOLOGIES FOR USE IN LIVER CELL THERAPIES OF
HUMAN HEPATIC STEM CELLS**

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ABSTRACT

RACHAEL ANN TURNER: Grafting Technologies for Use in Liver Cell Therapies of Human Hepatic Stem Cells
(under the direction of Lola M. Reid)

While the healthy liver is capable of rapidly regenerating after acute injury, patients with severe liver disease can undergo acute liver failure. The only widely used treatment for severe damage and failure is liver transplantation, but the paucity of available donor organs and the overwhelming number of patients waiting for transplants prompts a need for the development of alternative therapeutic strategies. Current therapies primarily introduce mature donor cells into hosts via cell suspension delivered through a vascular route; this results in inefficient engraftment, the engraftment of cells at ectopic sites, and can lead to complications with emboli formation and the need for immunosuppression. Sourcing of the cells to be transplanted is difficult, due mostly to the scarcity of available normal human tissue, in addition to poor cryopreservation methods, forcing one to obtain and utilize freshly isolated cells whenever available. Key problems associated with liver regeneration can be solved using tissue engineering themes which take advantage of the proliferative capacity of the stem cell population, along with its associated matrix. These strategies have been applied to three key facets needed for clinical programs in liver cell therapies: cryopreservation, 3-dimensional (3-D) cultures, and grafting technologies for transplantation of the cells.

The optimization of cryopreservation methods for stem cell banking was first achieved through the use of hyaluronan (HA) hydrogels. Isotonic medium supplemented with small amounts of HA (0.05 or 0.10%) improved cell attachment and colony formation due at least in part to increased expression of key cell adhesion factors in the stem and progenitor cell populations. The culturing and expansion of hHpSCs in an *ex vivo* three-dimensional (3-D) environment was achieved using hyaluronan hydrogels mixed with other matrix components found in the liver's stem cell niche, in combination with a tailored serum-free medium. Cell aggregates formed within the HA hydrogels, remained viable, and demonstrated a stable stem cell phenotype after weeks of culture. Finally, culturing conditions were utilized *in vivo* to improve methods of cell transplantation through the incorporation of grafting strategies after hepatic injury. These strategies were compared with current methods for transplantation of liver cells, which resulted in scattered aggregates within the liver and loss of significant numbers of cells to other locations. By contrast, grafting strategies provided a suitable scaffold which allowed all transplanted cells to remain within the liver and dramatically improved engraftment and expansion without evidence of emboli formation. The techniques developed here have improved current alternative methods available for the treatment of liver failure and are preclinical, but are expected to rapidly translate to therapeutic uses.

This dissertation is dedicated to the memory of my dear friend Stephanie Chance. Years ago, you were the one who convinced me I was ready for graduate school, and that I had the courage to move across the country to do so. You are by far one of the bravest and most selfless people I know. I am so honored to have had you as a part of my life.

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TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xvi
CHAPTER I	1
INTRODUCTION	1
Motivation	1
Objectives	3
CHAPTER II	5
HUMAN HEPATIC STEM CELL AND MATURATIONAL LIVER LINEAGE BIOLOGY	5
Prologue	5
Abstract	7
The Liver's Maturational Lineages	8
General Comments on Lineages	8
Known Maturational Lineage Stages found within Human Livers	13
Regulation of the Parenchymal Cell Lineages	28
Paracrine Signaling between Epithelial-Mesenchymal Partners	28
Regulation by Mechanical Forces	30

Feedback Loop Signals and forms of Liver Regeneration.....	33
Relevance of Cell Sources and Feedback Loop Signals to Clinical Programs	36
Liver Cancer Stem Cells	38
Conclusions.....	39
CHAPTER II.....	41
THE FUTURE OF CELL TRANSPLANT THERAPIES: A NEED FOR TISSUE GRAFTING.....	41
Abstract	42
Introduction.....	43
Cell Sourcing and Requisite Cells	45
Grafting Materials.....	46
Grafting Methods	47
Conclusions.....	50
CHAPTER IV	52
CRYOPRESERVATION OF HUMAN HEPATIC STEM/PROGENITOR CELLS- EFFECTS OF HYALURONANS ON THE CELLS AND THEIR ADHESION MOLECULES	52
Abstract	52
Introduction.....	54
Studies on Liver Cells	57
Methods.....	58
Liver sourcing and processing	58
Hepatic Stem Cell Culture Conditions.....	59
Freezing Conditions	60
Freezing Rate	62
Plating on Collagen III coated plates	62

Viability	63
Colony Counting.....	63
Quantitative Real Time PCR (qRT-PCR).....	63
Immunohistochemistry.	64
Microscopy	65
Statistical analysis.....	66
Results.....	66
Effect of freezing conditions and hyaluronans on cell viability and colony counts	66
Effect of hyaluronans on genetic expression of cell adhesion molecules and immunohistochemistry of adhesion proteins.	67
Discussion.....	70
Conclusions.....	74
CHAPTER V	75
MAINTENANCE OF HUMAN HEPATIC STEM CELL PHENOTYPE IN THREE DIMENSIONAL HYALURONAN CULTURES	75
Abstract.....	75
Introduction.....	76
Materials and Methods.....	82
Liver Sourcing and Processing.	82
Hepatic Stem Cell Culture Conditions.....	83
HA matrix Preparation.....	84
Cell matrix culture conditions.....	84
Viability Assay.....	85
Histology and Sectioning.....	85
Immunohistochemistry.	86

Confocal Microscopy.....	86
Analysis of secreted protein production.	87
Quantitative Real Time PCR (qRT-PCR).....	88
Results.....	88
Human hepatic stem cells remain viable and maintain phenotype for weeks in HA hydrogels.	89
Functional Assays of Hepatic Metabolism for hHpSCs seeded in hydrogels.....	91
Discussion.....	93
CHAPTER VI.....	96
LOCATION DETERMINATION OF TRANSPLANTED HEPATIC PROGENITORS USING POSITRON EMISSION TOMOGRAPHY (PET) AND LUMINESCENT IMAGING.....	96
Abstract.....	96
Background.....	97
Results and Discussion.....	98
Methods.....	102
Media and Solutions.	102
Human fetal liver processing.	103
Mouse liver processing.	103
Lentiviral construct and transfection.	104
Mice.	105
In vivo Engraftment.	105
IVIS optical Imaging.....	106
Statistical analysis.....	107
CHAPTER VII.....	108

<i>IN VIVO</i> GRAFTING OF HUMAN HEPATIC PROGENITORS INTO IMMUNOCOMPROMISED MURINE HOSTS.....	108
Abstract.....	108
Introduction.....	110
Methods.....	112
Liver Sourcing and Processing.	112
Hepatic Stem Cell Culture Conditions.....	113
HA matrix Preparation.	114
Mice.	115
In vivo engraftment.....	115
Optical Imaging.	116
Analysis of secreted protein production.	116
Histology.....	117
Results.....	117
Discussion.....	123
CHAPTER VIII	126
CONCLUSIONS.....	126
Conclusions.....	126
Recommendations for Future Research.....	128
REFERENCES	131

LIST OF TABLES

Table 1. Zonal Distribution of Cellular Subpopulations.....	9
Table 2. Intrahepatic Zonation of Functions.....	10
Table 3. Phenotypic Profiles of Multipotent Cell Populations in Human Livers	18
Table 4: Primer sequences used for quantitative RT-PCR.	64
Table 5: Antibodies and respective positive control cell lines used for immuno- histochemistry.	65
Table 6: Antibodies and respective positive control cell lines used for immuno- histochemistry.	86
Table 7: Primer sequences used for quantitative RT-PCR.	88

LIST OF FIGURES

Figure 1. Schematic image of liver, the biliary tree and pancreas and their connections with the duodenum. The blue stars indicate sites at which there are high numbers of peribiliary glands, the stem cell niches of the biliary tree.....	14
Figure 2: Schematic image of intrahepatic maturational lineages.....	15
Figure 3: Human hepatic stem cell and hepatoblast phenotypes in vivo. a,b) EpCAM expression in fetal liver: EpCAM expressed in the ductal plate is not only at the cell surface but also in the cytoplasm. EpCAM expressed in the hepatoblasts is specific to the cell surface. d,e) EpCAM expression in adult liver: One end of the Canal of Hering connects to the bile duct, the other end connects to hepatoblasts (arrow), indicating that the hepatoblasts are derived from primitive hepatic stem cells harbored in Canals of Hering. c) Double staining for CK-19/AFP and f) Ep-CAM/AFP of human fetal liver in the portal triad area and analyzed by confocal microscopy. CK-19 (c, green) is expressed not only by remodeling ductal plate but faintly expressed by some of the hepatoblasts. Ep-CAM (f, green) is detected in all the parenchymal cells and biliary epithelial cells forming bile duct and ductal plate (DP). AFP(red) is expressed by hepatoblasts throughout the fetal liver and undetectable in the ductal plate. (PT: Portal triad; DP: Ductal Plate).....	21
Figure 4: Human hepatic stem cell and hepatoblast phenotypes in culture. a) Morphology of human hepatic stem cells and b) human hepatoblasts in culture on plastic. c) Albumin staining of human hepatic stem cells, transitioning to hepatoblasts. d) hepatic stem cells stained with NCAM (green) and hepatoblasts stained with ICAM (red).....	21
Figure 5: Schematic image indicating the Coordinate Maturation of the Epithelia (Parenchymal cells) and their Mesenchymal Partners and some of the identified extracellular matrix components found at the particular lineage stages. Not shown in the figure are the soluble signals that also are lineage dependent. Some of those identified and that are lineage dependent are: hepatic stem cells are LIF, IL-6, IL-11, and acetylcholine; hepatoblasts are HGF, EGF, bFGF, IL-6, IL-11, and acetylcholine; hepatocytes are HGF, EGF, bFGF, T3, glucagon, and hydrocortisone; cholangiocytes are VEGF, HGF, bFGF. and acetylcholine.	30
Figure 6: Methods for grafting to different target tissues, including implantable grafts, injectable grafts, and patch grafts. Dependent on the diseased tissue type, one method may be more appropriate than another.	50
Figure 7: Schematic of cryopreservation and thawing methods.	61

Figure 8: Controlled rate freezing program minimizes liquid-ice phase entropy preventing internal ice damage and allows for repeatable freezing. A) Graph shows chamber temperature in relation to sample temperature (10% DMSO). B) freezing program rates used for Cryomed 1010 system.....	62
Figure 9: A) Cell Viability % of cryopreserved fetal hepatic cells post-thaw. B) Colony counts after 3 weeks of culture for each condition, normalized to fresh samples. Results are reported as mean \pm standard error of the mean. KM= Kubotas Medium with 10% DMSO and 10% FBS. CS10=cryostor, CS10+sup=cryostor10 with KM supplements. 0.05% and 0.10% refer to the HA% supplemented in each sample.....	67
Figure 10: Relative mRNA expression normalized to GAPDH expression. Mean \pm standard error of the mean. Significance * $p > 0.05$ to Fresh samples. KM= Kubotas Medium with 10% DMSO and 10% FBS. CS10=cryostor, CS10+sup=cryostor10 with KM supplements. 0.05% and 0.10% refer to the HA% supplemented in each sample.....	69
Figure 11: Cryopreserved hepatic progenitor cells after 24hr attachment on collagen III. CS10=cryostor, CS10+sup=cryostor10 with KM supplements. 0.05% and 0.10% refer to the HA% supplemented in each sample.....	70
Figure 12: hHpSCs in HA hydrogels at 21 days in culture. Green Calcein AM fluorescence shows viable cells.	89
Figure 13: Gene expression of hepatic stem cell phenotype markers. Expression levels are normalized to GAPDH expression, and fold changes are normalized to initial expression in colonies. * denotes $p < 0.05$ significance between experimental condition and initial colony expression. ** denotes $p < 0.05$ significance between experimental condition and initial colony expression as well as significant expression between the two experimental conditions.....	90
Figure 14: expression of EpCAM and NCAM in three-dimensional HA colony sections.....	91
Figure 15: functional assays of hepatic function over time. A) albumin, B) Transferrin, and C) Urea in three-dimensional hyaluronan culture over time for levels are normalized per cell.....	92
Figure 16: PET imaging of FHBG concentration activity in murine hosts injected via vascular route with human hepatic stem cells infected with TK. Positive signal is seen in liver, spleen, lungs, and kidney at 22 and 85 days post-injection.....	99
Figure 17: Transplantation of cells in murine livers . Injected cell suspension at a) 24 hrs and b) 72 hrs. c) control- no cells. Injectable cell graft at d) 24 hrs, e) 72 hrs, and f) 2 weeks post-transplantation.....	101

Figure 18: Lentiviral vector used to express thymidine kinase in implanted cells. 105

Figure 19: Total flux signal captured by luciferin expressing transplanted cells grafted with hyaluronans versus injected as a cell suspension in healthy and CCl4 induced liver injury models. Flux readings are normalized to control animals receiving no cell transplant..... 118

Figure 20: in vivo real time imaging of luminescent signal produced by luciferin-producing cells both grafted with hyaluronans versus injected as a cell suspension. 119

Figure 21: Serum human albumin at day 7 post-transplantation in grafted versus cell suspension in both healthy and CCl4 liver injury models..... 120

Figure 22: Controls for Histology of Human Transplanted Cells. A) and B) Control- no transplant. C) positive human fetal liver for human albumin and D) human fetal liver, no primary antibody staining..... 121

Figure 23: Histology of Human Transplanted Cells. Transplanted human hepatic progenitor cells stain positive for human albumin. Day 7 post-transplant. A-D) Grafting using Hyaluronan Hydrogels. E-H) Cell suspension. 122

Figure 24: electrospun silk patch for external patch grafting. 130

LIST OF ABBREVIATIONS

With respect to specific cellular subpopulations, the species of origin is indicated by a small letter in front of the abbreviation (r = rat; m=mouse; h= human)

AFP: α -fetoprotein

α SMA: α -smooth muscle actin

GABA: γ -aminobutyric acid

ALB: Albumin

ABAT: Apical bile acid transporter

CCl₄: Carbon tetracholoride

CS-PG: Chondroitin sulfate proteoglycan

CIII: collagen III

CFTR: Cystic fibrosis transmembrane conductance regulator

CK: Cytokeratin

DS-PG: Dermatan sulfate proteoglycan

EpCAM: Epithelial cell adhesion molecules

HA: Hyaluronic Acid(also referred to as hyaluronans)

HBs: Hepatoblasts,

HpSCs/HpSTCs: Hepatic stellate cells

HpSCs: Hepatic stem cells

HS-PG: Heparan sulfate proteoglycan

HP-PG: Heparin proteoglycan

ICAM: Intercellular adhesion molecules

IHH: Indian Hedgehog

KDR: Kinase insert domain receptor

Lam: Laminin

MHC: Major histocompatibility complex

MRP: Multidrug resistance associated proteins

MDR: Multidrug transporter

NCAM: Neural cell adhesion molecule

PG: Proteoglycan

SHH: Sonic Hedgehog

TGF- β : Transforming growth factor beta

VCAM: Vascular cell adhesion molecule

VEGFr: Vascular endothelial growth factor receptor

vWF: von Willebrand factor

CHAPTER I

Introduction

Motivation

As one of the most complex organs in the body, the liver is responsible for maintaining numerous functions, including toxin removal, production of bile and hormones, regulation of nutrients, and the synthesis of serum proteins. After acute injury, the liver is able to regenerate rapidly within days to a few weeks. However, patients whose disease is too severe can undergo liver failure. According to the Organ Procurement and Transplantation Network (www.optn.org), over 30,000 patients die annually from liver failure in the United States alone. Currently, the only widely used treatment for severe damage and failure is liver transplantation. However, the paucity of available donor organs has the consequences of leaving over 18,000 patients on the US waiting list and thousands more unable to get onto the list. With this overwhelming number of patients waiting for transplants, there is a great need for the development for alternative therapeutic strategies. Two such therapies being developed are transplantation of liver cells, currently as cell suspensions into patients, and bioartificial livers that can be used as assist devices.

Essential in developing alternative therapies is understanding and utilizing the liver's biology in terms of maturational lineage biology. The liver's maturational lineages are comprised of both the parenchymal and mesenchymal cells and are regulated by paracrine

signals produced by both subpopulations. The maturation of the two is paralleled by changes in the chemistry of the paracrine signals. The Reid lab is working on both therapeutic options making use of subpopulations of human liver cells with extraordinary expansion potential: human hepatic hHpSCs and their immediate descendents, hHBs, and committed progenitors. Extensive research has also been done on identifying the paracrine signals, comprising the matrix components and the soluble signals, found within the stem cell niche and between the parenchymal and mesenchymal cell partners during maturation to adult fates. The combination of the lineage-stage specific parenchymal and mesenchymal cell partners and their unique set of paracrine signals are ideal for tissue engineering applications.

Another development that has proven important is a method of culturing hHpSCs and hHBs in a three-dimensional (3-D) environment by using hyaluronan hydrogels as a base scaffold. Hyaluronans are present in all stem cell niches including those for liver. They are being used as a base condition for culture, cryopreservation and grafting technologies that collectively should greatly facilitate clinical and commercial programs utilizing liver cells as well as for basic research in mechanisms in stem cell biology and tissue engineering.

Current methodologies for cell therapies introduce donor cells into hosts via a vascular route. This works well for hemopoietic cells that float and have mechanisms of homing to appropriate sites, but it does not work well for cells from solid organs. The reports in the literature indicate that there is inefficient engraftment of cells in the appropriate location, and engraftment of cells at ectopic sites, that is those other than the liver (e.g. lung), as well as emboli formation. Grafting methods are being developed that combine cells within an appropriate matrix and hormonal environment to provide an optimal starting environment for survival, expansion and vascularization after transplantation.

Additionally, the supply of human hepatic cells depends on the availability of liver tissues from resections and rejected organs from organ donation programs. This supply is extremely limited and becomes available at unpredictable times. Methods for cryopreservation and long-term storage of isolated cells is vital in facilitating full use of the cells, thereby optimizing this scarce resource. An effective method of cryopreserving hHpSCs with consistent viability and attachment is needed for use in both research and clinical applications. In the last 20 years, multitudes of studies have been performed to establish a feasible freezing protocol on multiple tissue types from diverse species. No standard protocol has emerged, though several factors have been identified as being important for successful protocol. In combination with the protective characteristics of hyaluronan hydrogels, an effective method of cryopreservation for hHpSCs is being developed.

Objectives

Given the current limitations of liver disease therapies, improvements in cell cryopreservation and expansion, *ex vivo* bioreactors, and *in vivo* cell transplants can have huge benefits. The studies of this dissertation utilized the proliferative capacity of the stem cell population, along with its associated matrix components, to solve key problems associated with liver regeneration using tissue engineering themes. This first goal involves the optimization of cryopreservation methods for stem cell banking through the use of multiple variables found essential and with the addition of hyaluronan hydrogels. Secondly, the culturing and expansion of hHpSCs in hyaluronan hydrogels mixed with a combination of other matrix components *ex vivo* is being explored. Finally, these cultures can be utilized

in vivo to establish grafting methods that are being tested in immunocompromised murine hosts with and without hepatic injury. By embedding the cells into a microenvironment that mimics the liver's stem cell niche, current therapies can be vastly improved. These grafting technologies are explored and developed in this dissertation.

CHAPTER II

Human Hepatic Stem Cell and Maturation Liver Lineage Biology

Prologue

Hepatic stem cells and maturational lineage biology of the liver are central themes in my studies. This paper was published in *Hepatology* 2011, 53(3), p1035-1045, and is reprinted with permission from John Wiley and Sons, License # 2624911209300, and summarizes this maturational lineage biology, both intra- and extra-hepatically. Recognition of maturational lineage biology and its regulation by multiple mechanisms offers new understandings of liver biology, pathologies, and strategies for regenerative medicine. As a comprehensive review, additional authors also contributed to the writing of this manuscript. Oswaldo Lozoya prepared the sections on mechanical effects on cells. Yungfang Wang helped with sections on the biliary tree stem cells and on regulation of the cells by paracrine signaling. The sections on the biliary tree, biliary tree stem cells, and cholangiocytes were written and edited by Drs. G. Alpini, D. Alvaro, E. Gaudio and V. Cardinale.

Human Hepatic Stem Cell and Maturation Liver Lineage Biology

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Abstract

Livers are comprised of maturational lineages of cells beginning extrahepatically in the hepato-pancreatic common duct near the duodenum and intrahepatically in zone 1 by the portal triads. The extrahepatic stem cell niches are the peribiliary glands deep within the walls of the bile ducts; those intrahepatically are the canals of Hering in postnatal livers and that derive from ductal plates in fetal livers. Intrahepatically, there are at least 8 maturational stages from the stem cells located in zone 1 (periportal), through the midacinar region (zone 2), to the most mature cells and apoptotic cells found pericentrally in zone 3. Those found in the biliary tree are still being defined. Parenchymal cells are closely associated with lineages of mesenchymal cells, and the maturation of parenchymal and mesenchymal lineages is coordinate. Each lineage stage consists of parenchymal and mesenchymal partners distinguishable by their morphology, ploidy, antigens, biochemical traits, gene expression, and ability to divide. Lineage stages are governed by changes in chromatin (e.g. methylation), gradients of paracrine signals (soluble factors and insoluble extracellular matrix components), mechanical forces, and feedback loop signals derived from late lineage cells. Feedback loop signals, secreted by late lineage stage cells into bile, flow back to the periportal area and regulate the stem cells and other early lineage stage cells, in mechanisms dictating the size of the liver mass. Recognition of maturational lineage biology and its regulation by these multiple mechanisms offers new understandings of liver biology, pathologies, and strategies for regenerative medicine.

The Liver's Maturation Lineages

General Comments on Lineages

All tissues are organized with a stem cell niche containing stem cells and committed progenitors that give rise to daughter cells, maturing step-wise to adult cells, and transition to apoptotic cells [1]. The kinetics of the lineage and tissue turnover is tissue-specific and correlates inversely with the extent of polyploidy. Rapidly regenerating tissues have lineages with fast kinetics, and typically have only 5-10% polyploid cells. Newly recognized lineages are those associated with quiescent tissues (e.g. liver, pancreas, lung, kidney), with turnovers estimated to be months to years. The extent of polyploidy in these tissues in adults is from 20% to 95% [2].

Fetal and neonatal tissues are entirely diploid, and their transition to adult ploidy varies from species to species. In mice, it occurs within 3 weeks; in rats within 4 weeks; in humans by late teenage years [2]. With increasing age, the percentage of diploid cells steadily declines. In liver it increases from ~20% in young adults to over 50% in the elderly, a presumed variable in the regenerative capacity of the tissues [3].

The zonal distribution of the liver's known heterogeneity of functions has been described extensively in the past. In Table 1 and Table 2, we summarize these past studies on specific zones (periportal, midacinar, and pericentral) functions and include findings from more current literature to provide additional markers that are distributed zonally.

Table 1. Zonal Distribution of Cellular Subpopulations

Cellular Subpopulations	Stem Cell Niche*	Zone 1	Zone 2	Zone 3
Parenchymal Cell Populations				
Hepatic stem cells (HpSCs)	++	-	-	-
Hepatoblasts (HBs)	++	-	-	-
Committed progenitors	++	-	-	-
Hepatocytes	-	+++	+++	+++
Cholangiocytes	-	+++ (later lineage stages are extrahepatic)	-	-
Mesenchymal/Endothelial Cell populations				
Angioblasts	++	-	-	-
Endothelia cells (few, large fenestrations)	-	++	+	-
Endothelia cells (numerous, small fenestrations)	-	-	++	+++
Hepatic stellate cell (HpSTC) precursors	++ (with HpSCs)	-	-	-
HpSTCs	++ (with hHBs)	+++	+/- (+ in disease states)	- (+ in disease states)
Stromal cells	-	++ (most are extrahepatic biliary tissue)	-	-
Hemopoietic Cells				
Hemopoietic progenitors (CD34+)	++	-	-	-
Kupffer Cells (monocytes)	-	++ (phagocytosis)	++	+++ (cytotoxicity)
Lymphocytes	-	++	++	++
Pit cells (liver natural killer cells)	-	++	++	++

Table 2. Intrahepatic Zonation of Functions

	Protein or Activity/ mRNA	Zone 1 Periportal	Zone 2 Mid-Acinar	Zone 3 Perivenous
Carbohydrate Metabolism				
Phosphoenolpyruvate carboxykinase (glycogen from pyruvate)	Protein	+++		+
	mRNA	++++		+
Fructose-1,6-bisphosphatase	Protein	+		+
	mRNA	+++		+
Gluconeogenesis (from lactate, amino acids)		+++		+
Gluconeogenesis (from pyruvate)		+++		+
Glycogen Synthesis (from lactate)		+++		++
Glycogen Synthesis (from glucose)		++		+++
Pyruvate kinase Type L		+		+++
Glycolysis (glucose to pyruvate)			--	+++
Glucokinase (glycogen from glucose)	Protein	+		++
	mRNA	+		+
Amino Acid and Ammonia Metabolism				
Tyrosine aminotransferase		++	+	+
Serine dehydratase		+++		+
Glutamine synthetase (glutamine from ammonia; also from glutamate, α -oxoglutarate, ornithine)		--		+++
Ureogenesis (from ammonia, amino acid nitrogen via carbamoyl phosphate synthetase)		+++		+
Lipid Metabolism				
HMG-CoA reductase	Protein	+++		--

	mRNA	++		--
β-Oxidation		+++		+
Liponeogenesis Ketogenesis		+		+++
Cholesterol Biosynthesis		+++	--	--
Bile Acid Synthesis		--	+	++
Sulfation		+++		+
Glucuronidation		+		+++
Glutathione Content		+++		+
Glutathione-S-Transferases		+		+++
Glutathione Peroxidase		+++		+
Bile Acid Uptake	Na ⁺ dependent	+++	+	--
	Na ⁺ independ.	++		++
Uptake of many organic anions and cations in presence (with) or absence (without) of albumin	without	++	+	--
	With	++		++
Oxidative Energy Metabolism				
Succinate dehydrogenase		++		--
O ₂ uptake		++++		++
Mixed Function oxidation (NADPH cytochrome c reductase, epoxide hydrolase)		--	++	++++
Cytochrome P450 Isozymes				
Cyp 3A7		+++		-
CYP 1A, IIA, IIB, IIE, 3 ^a		-		++++
Specific Proteins				
Transferrin	Protein	+/-	++	+++
	mRNA	+++	+++	+++
ICAM-1 (sinusoidal endothelia and parenchyma associated with them)		++	+++	++++
NCAM (only in parenchymal cells in the stem cell niche)		+(niche)		--
EpCAM (hepatic stem/progenitors and		++	-	-

intrahepatic cholangiocytes)				
α -Fetoprotein (only in hepatoblasts)		++ (niche)		--
Albumin		++	++++ (full transcriptional regulation)	
Connexin 26		+++		+
Connexin 32		+		+++
Extracellular Matrix Components				
Collagen I		+		++++
Collagen III		++		++++
Fibronectin (tissue)		++	++	++++
Fibronectin (plasma)		--		++++
Heparin-PG		-		++++
Collagen IV		++++		--
Collagen V		++++		?
Collagen VI		++++		?
Collagen XVIII		+++		?
Laminin		++++		--
Hyaluronans (produced by hHpSTCs and endothelia)		++++		---
Chondroitin sulfate-PGs		++++	++	+
Heparan sulfate-PGs		++	++	++
Dermatan sulfate-PGs		++	++	+
*Stem cell niche: ductal plates (also called limiting plates) in fetal and neonatal livers; canals of Hering in pediatric and adult livers .				
++++ = strong signal; + = weak signal ; -- = no signal.				
Tables have been prepared from data in reviews on heterogeneity of functions in liver by Gebhardt[4] [see Figures 3 and 4 and Tables 2 and 3] and by Jungermann and Kietzmann[5] [see Figures 2, 3 and Tables S2 and S3] and from diverse, more recent studies [6-9]				

Extrahepatic Lineages in the Biliary tree. Multipotent stem cell populations have been identified recently in the peribiliary glands of the biliary tree, giving rise to liver, bile duct,

and pancreas under specific culture conditions or with transplantation *in vivo* [10, 11]. The antigenic and biochemical profiles of the biliary tree stem cell populations at different sites in the extrahepatic bile ducts are suggestive of multiple lineage stages with the most primitive ones being within the hepato-pancreatic common duct near to the duodenum (**Figure 1**). Later stages are found in the cystic duct and hilum. Related cells, possibly transit amplifying cells, are found within the gallbladder that does not have peribiliary glands. Further characterization of these cells should elucidate possible precursor-descendent relationships including if they are precursors to intrahepatic lineages. A review summarizing the extant knowledge of these newly discovered lineages is given elsewhere [11].

Known Maturation Lineage Stages found within Human Livers

This overview will focus on diploid subpopulations, particularly stem/progenitors for which less has been published. The extrahepatic lineages and the adult lineage stages are noted with brief descriptions of their phenotypic features. Schematic figures demonstrating the concepts of hepatic lineages can be seen in **Figure 1** and **Figure 2**. Currently, there is evidence for at least 8 intrahepatic lineage stages [12-17]. As efforts continue to characterize the liver's lineage biology, we assume that additional lineage stages will be identified.

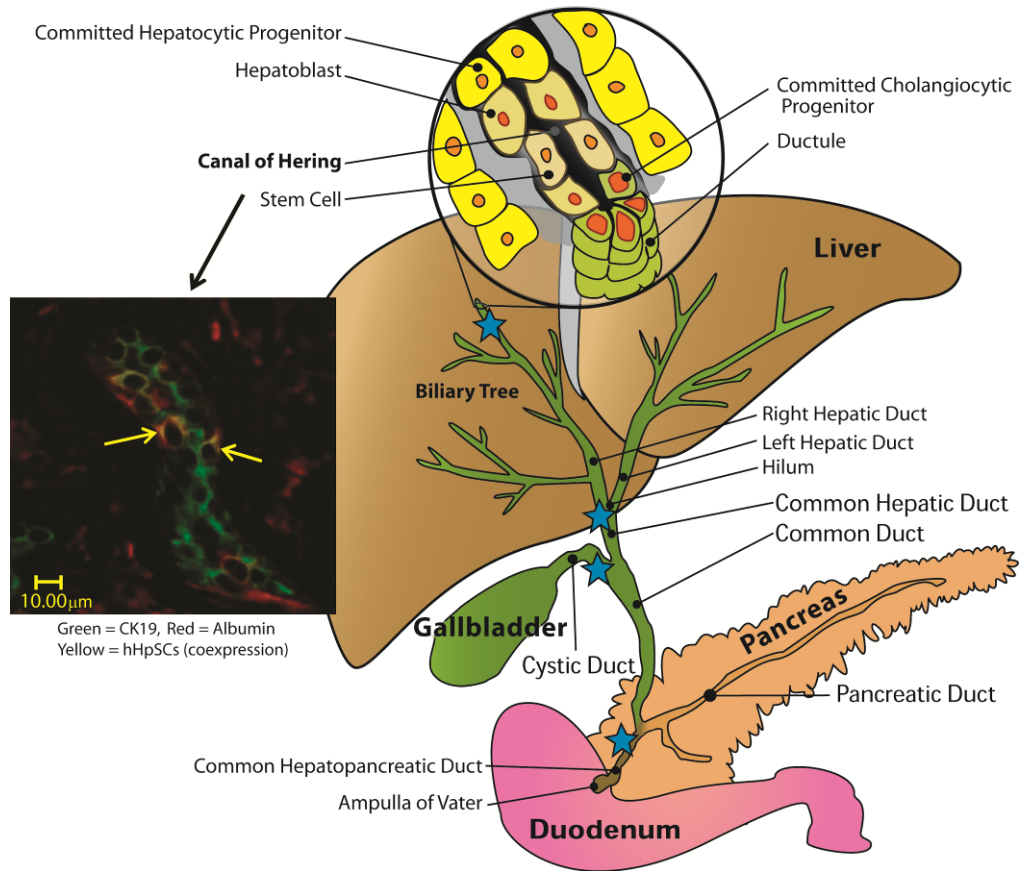


Figure 1. Schematic image of liver, the biliary tree and pancreas and their connections with the duodenum. The blue stars indicate sites at which there are high numbers of peribiliary glands, the stem cell niches of the biliary tree.

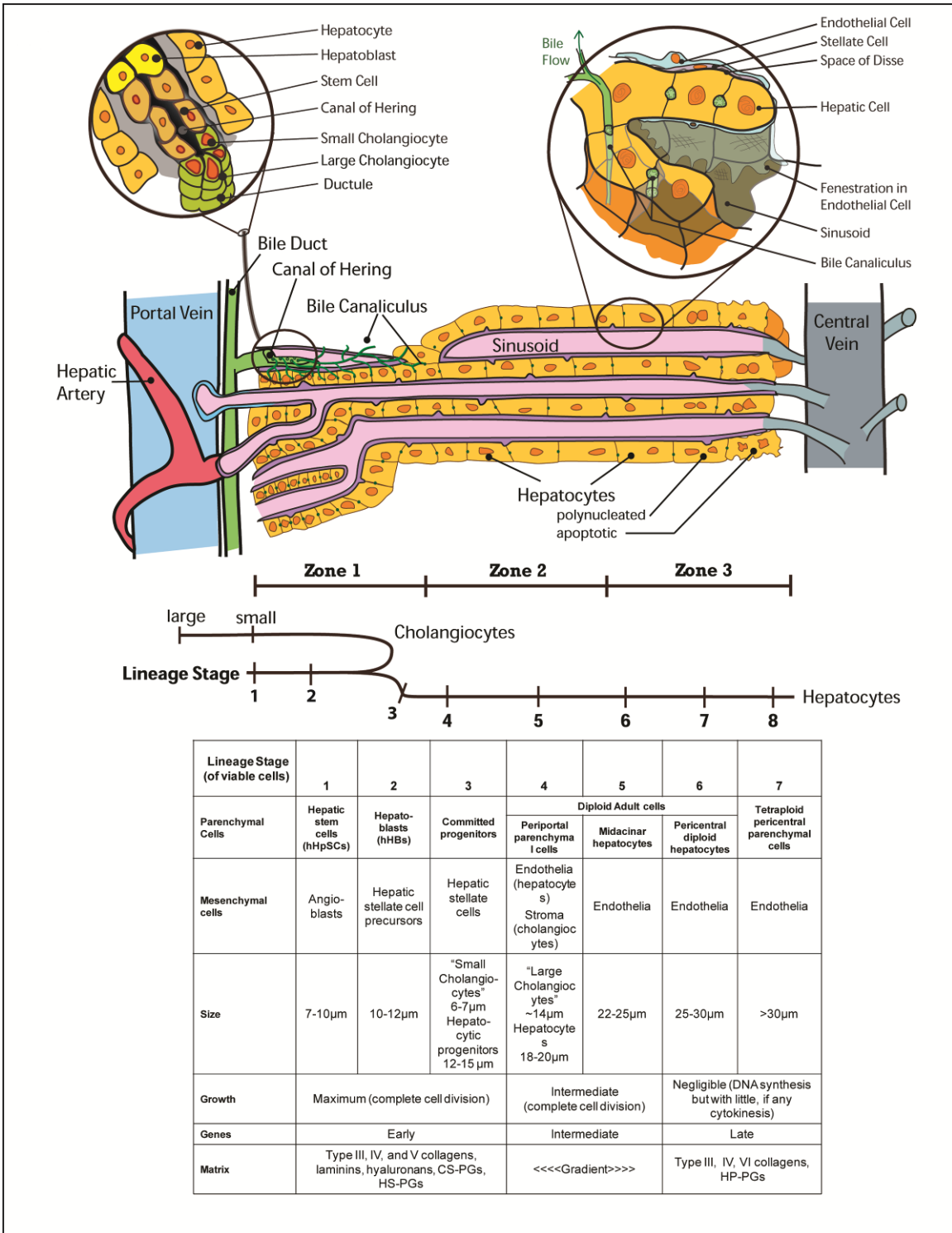


Figure 2: Schematic image of intrahepatic maturational lineages.

Intrahepatic Lineage-Stage 1. Human hepatic stem cells (hHpSCs) are multipotent stem cells located within the liver's stem cell compartment, the ductal plates of fetal and neonatal livers and canals of Hering in pediatric and adult livers [12, 18-23]. The compartment represents the anatomic and physiological link between the intralobular canalicular system of hepatocytes and the biliary tree and resides along an array of sites that project starlike from the portal tracts. The hHpSCs cells range in size from 7-10 μm in diameter and have a high nucleus to cytoplasmic ratio. They are tolerant of ischemia and can be found as viable cells in cadaveric livers for up to ~6 days after asystolic death [22, 24]. The hHpSCs form colonies in culture capable of self-replication [25] and of differentiation to mature cells in culture and *in vivo* [18, 26]. They constitute ~0.5-2% of the parenchyma of livers of all age donors. Their known antigenic profile comprises epithelial cell adhesion molecule (EpCAM), neural cell adhesion molecule (NCAM), CD133, CXCR4, SOX9, SOX17, FOXA2, cytokeratins (CK) 8/18/19, hedgehog proteins (Sonic and Indian), claudin 3, and with weak (if any) expression of albumin, MHC antigens, certain pluripotency genes (Nanog, KLF4, OCT4, and SOX 2), and **no** expression of α -fetoprotein (AFP), intercellular adhesion molecule (ICAM-1), P450s, markers for hemopoietic cells (e.g. CD34, 38, 45, 90, or glycophorin), endothelial cells (e.g. VEGFr, CD31, von Willebrand factor) or mesenchymal cells (e.g. CD146, desmin, vitamin A, CD105) [17, 18, 26]. C-kit (CD-117) has also been found in the liver's stem cell niches [19, 27, 28], but flow cytometric sorts for CD117+ cells selects for angioblasts [18, 26]. Therefore, it remains unclear if it is on the hHpSCs or on angioblasts closely bound to hHpSCs, so further studies are needed to clarify this point. The hHpSCs have pumps such as MDR1 that eliminate xenobiotics [29]. In addition, they express telomerase mRNA and have telomerase protein entirely localized within the nucleus [30].

Some proteins, such as CK19, are synthesized and found in punctuate form, but not converted to filaments [18]. Ability of the cells to form the filamentous form occurs at the next lineage stage, the hepatoblasts. Similarly, albumin is weakly synthesized (if at all) but is not packaged as it will be in the hepatoblasts and later lineage stages. This implicates lineage-dependent distinctions in how some proteins are processed post-transcriptionally and translationally.

The hHpSCs are readily isolated by immunoselection for cells doubly positive for [EpCAM+ NCAM+] from livers of all donor ages. In adult livers in which hepatoblasts are scarce, selection for EpCAM+ cells results in predominantly hHpSCs [18, 31]. They expand *ex vivo* if in a serum-free medium, “Kubota’s Medium”, designed for endodermal progenitor cell populations [32, 33] and if co-cultured with angioblasts, plated onto purified type III collagen, or embedded into loosely cross-linked hyaluronan hydrogels [26, 34]. If transplanted into immunocompromised hosts, they yield mature liver tissue. If cultured under distinct conditions (see below) they lineage restrict into hepatoblasts [26].

Intrahepatic Lineages-Stage 2. Hepatoblasts are diploid, bipotent cells giving rise to hepatocytic and cholangiocytic lineages and capable of enormous expansion potential, but not yet found to have self-replication ability [18, 26]. The requirements for their expansion overlap with but also include distinctions from those of the hHpSCs [26]. The gene expression profile comprises stem/progenitor cell genes (e.g. EpCAM, CXCR4, CD133, pumps such as MDR1 [29] that enables them to eliminate xenobiotics), usually faint or no expression of pluripotency genes (e.g. OCT4, Nanog, KLF4), and some genes unique to the hepatic fates (e.g. albumin, AFP). Hepatoblasts (hHBs) are the immediate descendents of the hHpSCs, are the liver’s probable transit amplifying cells, and the expression of AFP is a

signature feature. They are located just outside the stem cell niche proper, as a single cell or small aggregate of cells adjacent to or tethered to the ends of the ductal plates or canals of Hering [19]. These cells are larger (10-12µm) with higher amounts of cytoplasm and are found *in vivo* throughout the parenchyma in fetal and neonatal livers. They have telomerase protein within the nucleus and approximately 5X the telomerase activity of that in the hHpSCs and telomerase protein found both in the nucleus and in the cytoplasm [30]. CK14 and CK19 are found as filaments [19, 28, 35] and albumin localized in discrete packets cytoplasmically [18]. With donor age, the hHBs decline in numbers to <0.01% of the parenchymal cells in postnatal livers [18, 19]. They have been shown to expand during regenerative processes, especially those associated with certain diseases such as cirrhosis. Previously, these cells were referred to as “intermediate hepatobiliary cells of the ductular reactions”[36]; however, extensive characterization enabled us to replace the nomenclature with hepatoblasts [19]. A comparison of the phenotypic profiles of HpSCs and HBs can be found in **Table 3**, **Figure 3** and **Figure 4**.

Table 3. Phenotypic Profiles of Multipotent Cell Populations in Human Livers

Property	Human Hepatic Stem Cells (hHpSCs)	Human Hepatoblasts (hHBs)
Average diameter (measured by forward scatter in flow cytometric analyses of isolated cells)	7-9 µm	10-12 µm
Nucleus to cytoplasmic ratio	Highest observed of all parenchymal progenitor subpopulations evaluated	Intermediate between that in hHpSCs and mature parenchymal cells
Percentage of parenchymal cells [18]	0.5-1.5 % in livers of all donor ages and with minimal ischemia; percentages higher in ischemic livers	>80% (fetal livers) ~50% (neonatal livers) [percentages change rapidly day by day postnatally]

		<0.01% (adult livers)
Survival after cardiac arrest (tolerance for ischemia) [18]	Viable cells for several days after cardiac arrest	Viable for more than a day, but not as long as hHpSCs
Morphology of colonies <i>in vitro</i> [18]	Uniform; densely packed; look similar to ES cell colonies	Cord-like colonies interspersed with clear channels that are presumptive canaliculi
Evidence for Self-renewal [25]	Clonogenic expansion with stability of phenotype; doubling times of ~36 hours on plastic; can be passaged repeatedly; fastest doubling times (~20-24 hours) for hHpSCs on substrata of type III collagen	Significant expansion potential but not yet evidence for self-replication (under the conditions tested to date). Probable transit amplifying cells
Pluripotency [18]	Multipotent	Bipotent
Anaerobic metabolism (metabolomic studies) [34]	+++	+++
Conditions for clonogenic expansion [32]	Kubota's Medium plus feeders of angioblasts replaceable with type III collagen (monolayers) or hyaluronans into which is mixed type III collagen (3-D)	Kubota's Medium plus feeders of hepatic stellate cells replaceable with type IV collagen/laminin (monolayers) or hyaluronans into which is mixed type IV collagen/ laminin (3-D)
CD44H (hyaluronan receptor) [37]	High concentrations	High concentrations
Claudin 3 [31]	+++	Negative
Indian Hedgehog [12]	+++ Highest level in cells in the center of the colonies	++ Lower levels, but pattern of distribution is the same
Sonic Hedgehog [12]	++ Located at edge of cells; concentrated in cells at edge of colonies at sites of high concentration of angioblasts	+ Lower levels, but pattern of distribution is the same

Patched (Hedgehog receptor) [12]	+++ Found in all cells and in colonies throughout the colony	++ Levels lower, but still evident
Telomerase [30]	+ mRNA encoding telomerase and the protein found in nucleus. No telomerase protein in the cytoplasm	+++ mRNA encoding telomerase and the protein found in nucleus; with differentiation, increasing numbers of the cells have it in the cytoplasm; 5X higher activity than in hHpSCs
P450s [31]	Negative for all assayed	P450 A7 but not late forms of P450s
CK 8 and 18 [31]	++	++
CK 19 [18, 31]	++ (not in filament form)	++ (filaments evident)
E-cadherin [18]	++	++
EpCAM [18, 19]	+++ (throughout the cells)	++ (plasma membrane)
NCAM /ICAM-1 [18, 19]	++ /-	-/++
Albumin [18]	±	++
α-fetoprotein [18, 19]	Negative	+++
*Mesenchymal Markers	Negative	Negative
**Angioblasts/ Endothelial cell Markers	Negative	Negative
***Hemopoietic markers	Negative	Negative

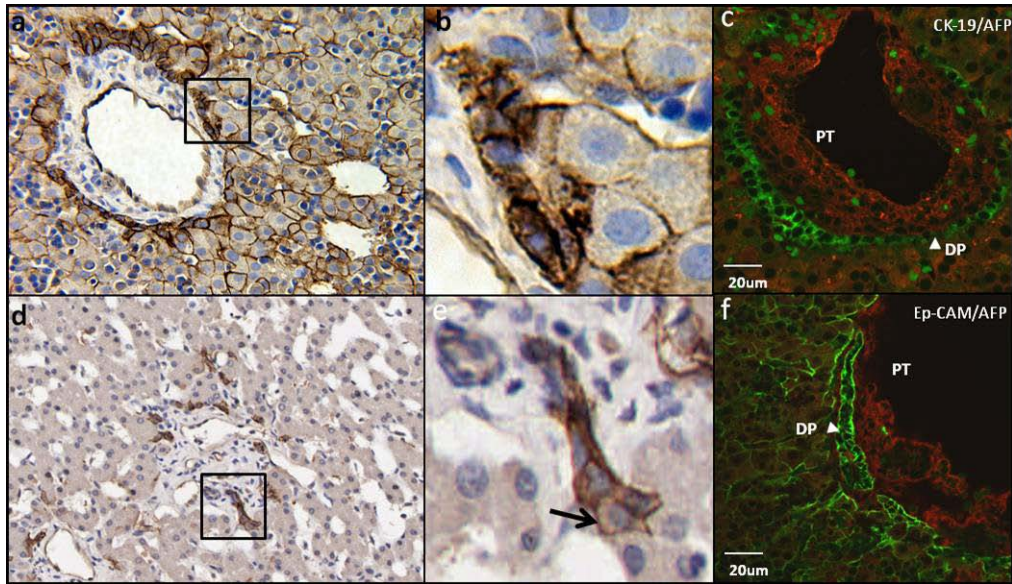


Figure 3: Human hepatic stem cell and hepatoblast phenotypes in vivo. a,b) EpCAM expression in fetal liver: EpCAM expressed in the ductal plate is not only at the cell surface but also in the cytoplasm. EpCAM expressed in the hepatoblasts is specific to the cell surface. d,e) EpCAM expression in adult liver: One end of the Canal of Hering connects to the bile duct, the other end connects to hepatoblasts (arrow), indicating that the hepatoblasts are derived from primitive hepatic stem cells harbored in Canals of Hering. c) Double staining for CK-19/AFP and f) Ep-CAM/AFP of human fetal liver in the portal triad area and analyzed by confocal microscopy. CK-19 (c, green) is expressed not only by remodeling ductal plate but faintly expressed by some of the hepatoblasts. Ep-CAM (f, green) is detected in all the parenchymal cells and biliary epithelial cells forming bile duct and ductal plate (DP). AFP (red) is expressed by hepatoblasts throughout the fetal liver and undetectable in the ductal plate. (PT: Portal triad; DP: Ductal Plate)

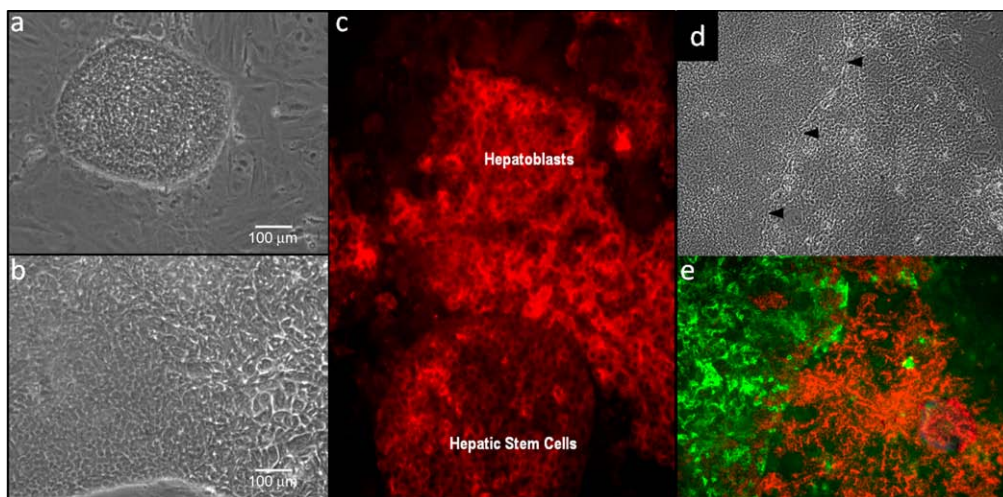


Figure 4: Human hepatic stem cell and hepatoblast phenotypes in culture. a) Morphology of human hepatic stem cells and b) human hepatoblasts in culture on plastic. c) Albumin staining of human hepatic stem cells, transitioning to hepatoblasts. d) hepatic stem cells stained with NCAM (green) and hepatoblasts stained with ICAM (red).

The antigenic profile of hHBs overlaps in part with that of the hHpSCs [17, 18, 28] with shared markers being cytokeratins(CK) 8/18 and 19, hedgehog proteins (Sonic and Indian), and **no** expression late P450s (e.g. P450-3A), markers for hemopoietic cells (e.g. CD34,38,45,90,or glycoporphin), endothelial cells (e.g. VEGFr,CD31,von Willebrand factor) or mesenchymal cells (e.g. CD146,desmin,vitamin A,CD105). The changes comprise a reduction in levels of EpCAM restricting it primarily to the plasma membrane surface, a switch from NCAM to ICAM-1, elevated expression of albumin, expression of early P450s (e.g. P450-A7), of CK7, and very strong expression of AFP. The AFP expressed by hHBs is distinct from one expressed by hemopoietic progenitors only by the sequences expressed in exon 1, a probable clue of mesendoderm to endoderm differentiation [38].

The hHBs can be purified from liver cell suspensions by immunoselection for cells that are doubly positive for [EpCAM+, ICAM-1+]. They can be expanded in culture in Kubota's Medium and on feeders of hepatic stellate cells [26, 39] that can be replaced, in part, with substrata of purified type IV collagen mixed with laminin or 3-dimensionally in cross-linked hyaluronans, ideally into which are mixed type IV collagen and laminin [26, 37].

Intrahepatic Lineages-Stage 3. Committed progenitors are diploid, unipotent, immature cells ~12-15 μm in diameter. These precursors give rise to only one adult cell type. They no longer express some of the stem cell genes (e.g. EpCAM, NCAM) or the pluripotency genes, and express only faintly other genes strongly expressed by the stem cells such as CD133/1 or Hedgehog proteins. They express either hepatocytic markers or biliary markers, but not both. They are found in large numbers in fetal and neonatal tissues, rarely observed in normal adult tissues, and are observed again in large numbers in chronic liver

diseases (viral, alcoholic and non-alcoholic fatty liver diseases, autoimmune hepatitis, cholangiopathies) [40, 41].

Committed hepatocytic progenitors, also called intermediate hepatocytes, express albumin, glycogen, and the enzymes associated with glycogen synthesis (e.g. glucose-6-phosphate); they do not express biliary markers, such as CK19, and minimal (if any) EpCAM. If AFP is expressed, it is found only as faint or weak expression. Cells are located *in vivo* in the liver plates between the hHBs and the diploid adult hepatocytes. The numbers are very small except in disease states.

“Small cholangiocytes” are hypothesized to be committed biliary progenitors and co-localize with the hHpSCs in the stem cell niche, lining the canals of Hering, intrahepatic bile ducts and bile ductules with an internal diameter of less than 15 μm [42]. The canals of Hering link directly to bile ductules which may or may not traverse the limiting plate, and thus may have an intralobular segment (periportal) in addition to their intraportal location. Small cholangiocytes are diploid, are ~6-8 μm in diameter, have a cuboidal shape, a high nucleus to cytoplasm ratio, and inconspicuous endoplasmic reticulum [43, 44]. In human and rodent livers, small cholangiocytes express high levels of the anti-apoptotic proteins, annexin V and bcl2 (B-cell lymphoma 2 protein). At a functional level, they express various genes, including receptors for endothelin receptors type A (EDNRA) and type B (EDNRB), endogenous opioid peptides, insulin, histamine (H_1), acetylcholine (M3), and α -1-adrenergic agonists, aquaporin 4, but not the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, secretin or somatostatin receptor, nor the Na^+ -dependent bile acid transporter, ABAT (apical bile acid transporter). CFTR (cystic fibrosis transmembrane conductance regulator) was found in human, but not rodent, small cholangiocytes [42]. Finally, small cholangiocytes exhibit the *de novo* expression of the

apical sodium-dependent bile acid transporter during chronic feeding with the bile salts, taurocholate, and tauroolithocholate, suggesting a role in the cholehepatic recirculation of bile salts in conditions of overload [45].

Intrahepatic Lineages-Stages 4-6. Diploid adult cells are able to undergo complete cell division and are the only adult parenchymal cells with significant proliferative capacity under all known *in vitro* or *in vivo* conditions. There are two exceptions with respect to findings *in vivo*: tyrosinemia [46] or massive loss of most mature parenchymal cells (e.g. >80%) due to a transgene [47]. It is hypothesized that tyrosinemia and/or loss of most of the mature parenchyma by such experimental manipulations causes reprogramming (demethylation of the chromatin) of the transplanted cells, a process that would restore cytokinesis. Thus, the controversial claim that all adult hepatocytes are stem cell-like [48] is unproven and based on findings relevant to a unique disease or experimental condition.

The zone 1, mature hepatocytes can form colonies from 6-7 rounds of division within 2-3 weeks but have limited capacity to be subcultured [32, 49, 50]. As one progresses in the lineage stages towards the central vein, the ability of any diploid subpopulations to undergo complete cell division (that is, with cytokinesis) is minimal. The proportion of diploid mature cells in young adults is only ~3% in mice, ~10% in rats, and the majority of the parenchyma in humans.

Intrahepatic Lineage-Stage 4. Periportal parenchymal cells (zone 1) are comprised of “small (diploid) hepatocytes” [50] and intrahepatic biliary epithelia, or “large cholangiocytes” [42]. The hepatocytes are ~18 μm and the large cholangiocytes are ~14 μm in diameter. The hepatocytes form plates or cords of cells bound on their lateral borders to each other by a mix of lateral matrix components (cell adhesion molecules, proteoglycans),

tight junctions (cadherins), and gap junctions (connexins) [51-53]. The proteoglycans on the lateral borders are known to regulate multiple aspects of gap junction functions as well as transcription of tissue-specific genes [53, 54]. In the center of the lateral border connection between two hepatocytes is the bile canaliculus, a region of undulating membrane studded with enzymes and pumps that transfer hepatocyte-derived products into bile in the canaliculus [42].

Hepatocytes are unique among epithelia in having two basal surfaces, bound to extracellular matrix components (collagens, proteoglycans, adhesion molecules) in the Space of Disse, produced by the hepatocytes and their mesenchymal cell partners, endothelial cells and pericytes [7, 55-59]. Zone 1 metabolic activities (see **Table 2**) are highest in periportal hepatocytes. In brief, they produce factors and enzymes associated with gluconeogenesis, amino acid and ammonia metabolism, urea synthesis, and glutathione peroxidase [16].

Large cholangiocytes are columnar in shape and display a small nucleus and conspicuous cytoplasm. An abundant Golgi apparatus is observed between the apical pole and the nucleus, and rough endoplasmic reticulum is more abundant than small cholangiocytes [42]. *Large cholangiocytes line interlobular ducts located in the portal triads and all larger intrahepatic ducts.* They express CFTR and Cl⁻/HCO₃⁻ exchanger, aquaporin 4 and aquaporin 8, secretin and somatostatin receptors other than receptors for hormones and neuropeptides. In addition, they express the Na⁺-dependent bile acid transporter apical bile acid transporter ABAT(SLC10A4), multidrug transporters MDR3(ABCB4) and MDR1(ABCB1), and multidrug resistance associated proteins, MRP2(ABCC2) and MRP3(ABCC3) [60]. When large cholangiocytes are damaged by acute carbon tetrachloride (CCl₄) or GABA administration, small cholangiocytes proliferate, and acquire phenotypical

and functional features of large cholangiocytes [61, 62]. This suggests that the population of small cholangiocytes lining the canals of Hering and ductules may represent precursors of large cholangiocytes lining larger ducts. By using microarray methods, the integrated differential gene expression between small and large normal cholangiocytes demonstrate that the proteins related to cell proliferation tend to be highly expressed by small cholangiocytes, whereas large cholangiocytes express more functional and differentiated genes [63]. This is consistent with studies showing, either with bile duct injury due to CCl₄ and GABA administration or with bile duct regrowth following partial hepatectomy, that small cholangiocyte proliferation is activated presumably to repopulate bile ducts. These findings suggest that small cholangiocytes are less mature, have a high resistance to apoptosis, and have marked proliferative activities, while large cholangiocytes are more differentiated, contributing mainly to ductal bile secretion and absorption. Therefore, while hepatocytic cell lineages proceed from periportal areas toward the central vein, cholangiocytes proceed in the opposite direction from canals of Hering/bile ductules toward larger ducts.

Intrahepatic Lineages- Stage 5. Midacinar hepatocytes (Zone 2) are diploid in humans, tetraploid in rats, and 4-8 N in mice, ~22-25 µm in diameter, and located in the midacinar zone [2]. The strategies for studying zonation of functions, selective destruction of periportal or pericentral cells with detergents characterizing cell suspensions, are not able to give precise definition to the functions of zone 2 cells [64]. Recognition of some unique features of the midacinar parenchymal cells has emerged with immunohistochemical and *in situ* hybridization studies on sections of livers. The midacinar hepatocytes are the first stages to have peak levels of certain transcription factors regulating albumin enabling these cells to produce especially high levels of the protein [65, 66]. In addition, transferrin mRNA is

expressed in earlier lineage stages, but it does not translate to protein at detectable levels until zone 2 (midacinar), correlating with production of specific elongation factors associated with translation of transferrin mRNA to protein [31]. It is unknown whether this is true for other proteins. There must be distinctions in posttranscriptional and translational regulation of certain mRNAs for early lineage stage cells versus later ones, observations yet to be fully explored.

Intrahepatic Lineage-Stage 6. Pericentral diploid hepatocytes (zone 3) are found in small numbers in human livers, but in rats and mice there are none; all rodents have only polyploid cells in zone 3. The diploid parenchymal cells in humans decline with age in parallel with an increase in polyploidy. In culture, they are able to undergo DNA synthesis but with limited, if any, ability to undergo cytokinesis, and no capacity to be subcultured [33]. In addition to albumin, tyrosine aminotransferase, and transferrin, they also strongly express a number of the P450s that handle xenobiotic metabolism (e.g. P450-3Aa), glutathione transferases, and UDP-glucuronyl-transferases [67, 68]

Intrahepatic Lineage- Stage 7. Pericentral parenchymal cells (zone 3) can undergo DNA synthesis but are unable to undergo cytokinesis in all species [2]. In humans they are tetraploid; in rats they are octaploid; and in mice they are 16-32 N. They are much larger (>30 μm in diameter in human hepatocytes and up to 75 μm in rodents) due to the hypertrophy associated with polyploidy. They express high levels of the late genes including the late P450s, glutathione transferases, UDP-glucuronyl-transferases, glutamine synthetase and heparin proteoglycans [69, 70].

Intrahepatic Lineage- Stage 8. Apoptotic cells express various markers of apoptosis and demonstrate DNA fragmentation. Clustered near to these apoptotic cells are Kupffer cells, responsible, in part, for eliminating the dying parenchymal cells [69].

Regulation of the Parenchymal Cell Lineages

Paracrine Signaling between Epithelial-Mesenchymal Partners

Paracrine signaling is the primary form of regulation between parenchymal cells and their partner mesenchymal cells and represents the classic epithelial-mesenchymal relationship described by embryologists and developmental biologists since the 1930s. The epithelial-mesenchymal relationship's importance in development and in adult tissues has been thoroughly described in the primary literature and in many reviews and will not be presented here [71, 72]. Rather, the only new facet requiring recognition is that coordinate maturation of the parenchymal and mesenchymal cell partners occurs and is associated with lineage-dependent gradients of paracrine signals [26].

Within the stem cell niche, the hHpSCs are partnered with angioblasts and regulated by a set of paracrine signals. As hHpSCs mature through hepatocytic or cholangiocytic lineages, they are partnered by specific lineage stages of mesenchymal cells; each stage is defined by a unique set of paracrine signals. The intrahepatic lineages begin with hHpSCs/angioblasts giving rise to hHBs/precursors of stellate and endothelial cells. This splits to yield lineages of hepatocytes/endothelia and cholangiocytes/mature stellate cells progressing to cholangiocytes/myofibroblasts. The gradients of paracrine signals (soluble

signals and insoluble extracellular matrix components) govern the biological responses of the cells at each stage [71, 73].

Some sets of paracrine signals have been partially defined and have been used to establish parenchymal cells at a specific lineage stage in culture (**Figure 5**). For example, the stem cell niche has been found to contain type III collagen, a laminin form binding to $\alpha 6\beta 4$ integrin, hyaluronans and a minimally sulfated chondroitin sulfate proteoglycan(CS-PG) [26]. With hHBs, the signals change to type IV collagen, laminin binding to $\alpha \beta 1$, hyaluronans, more sulfated CS-PGs and forms of heparan sulfate-PGs. The hepatocytic lineage is associated with network collagens (e.g. type IV and VI) and forms of heparan sulfate-PGs (HS-PGs) with increasing sulfation ending, in zone 3, in heparin-PGs, HP-PGs. The cholangiocytic lineage is associated with fibrillar collagens (e.g. type I and type III) and forms of CS-PGs ending in highly sulfated forms, i.e. dermatan sulfate-PGs, DS-PGs [25, 26, 37]. The gradients in matrix chemistry are paralleled by ones of soluble signals, most being bound to various matrix components, particularly the glycosaminoglycans (GAGs) that are part of the PGs [74]. The chemistry of the matrix works synergistically with the soluble signals to dictate specific biological responses from the cells. Indeed, the soluble factors are biphasic, yielding mitogenic effects when complexed with the less sulfated proteoglycans and causing growth arrest and differentiation when complexed with the highly sulfated ones. These effects are mediated by classic signal transduction pathways complemented by the mechanical effects of the matrix (see below).

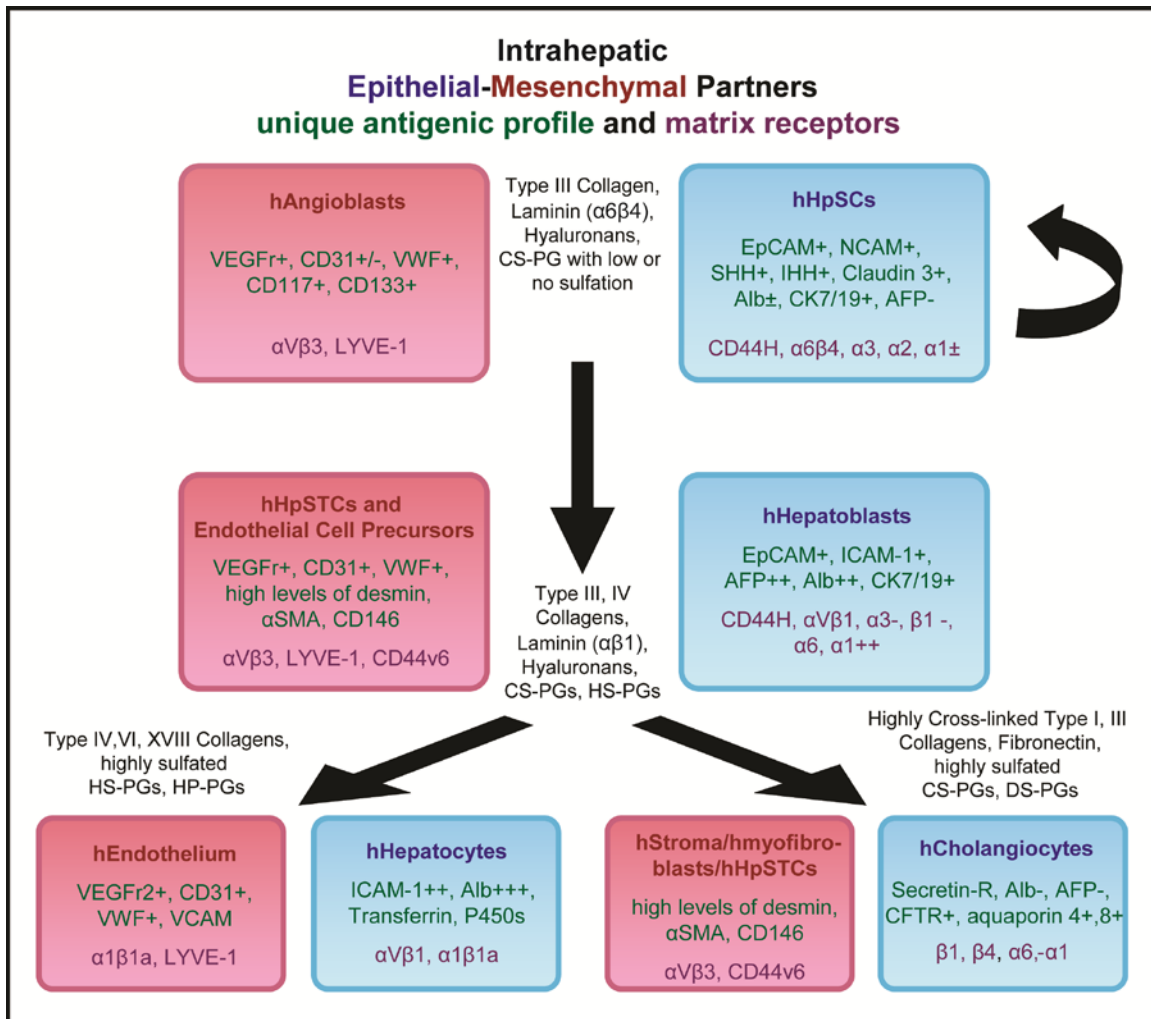


Figure 5: Schematic image indicating the Coordinate Maturation of the Epithelia (Parenchymal cells) and their Mesenchymal Partners and some of the identified extracellular matrix components found at the particular lineage stages. Not shown in the figure are the soluble signals that also are lineage dependent. Some of those identified and that are lineage dependent are: hepatic stem cells are LIF, IL-6, IL-11, and acetylcholine; hepatoblasts are HGF, EGF, bFGF, IL-6, IL-11, and acetylcholine; hepatocytes are HGF, EGF, bFGF, T3, glucagon, and hydrocortisone; cholangiocytes are VEGF, HGF, bFGF, and acetylcholine.

Regulation by Mechanical Forces

An important aspect of homeostatic regulation in liver and other organs (e.g. pancreas), is the effect of mechanical stimuli. Research on the mechanical forces contributing to differentiation has revealed that differentiation involves stiffness-dependent mechanisms [75] accompanied by loss of mechanical compliance in cells [76], and that embryos exhibit

phase ordering that parallels behavior in liquid mixtures [77-79]. The role of active flow induction by mechanical organelles, such as cilia, has been linked to differentiation patterns in embryos [80]. These physical principles, present in early development, are active also in mature tissues controlling tissue formation, turnover and maintenance of mechanically controlled mechanisms at all stages in life [81].

Mature tissues become specialized both through cellular differentiation mediated by their surrounding extracellular matrix and soluble signal composition. In general, cells require specific machinery to sustain forces that reach them. Differentiation occurs in cells in part due to gradients in the matrix chemistry synergizing with soluble signals during tissue development, but also because of differential mechanical properties of the matrix [82, 83]. As noted above, the maturational cellular lineages in adult liver are paralleled by distinct matrix gradients. That for the hepatocytes and their associated endothelial cell partners is soft, highly porous with parallels for that found in capillary beds. That for the cholangiocytes-stellate cell/stromal cell partners is highly cross-linked and more rigid. This defines a durotactic gradient [9, 84]. Findings indicate that stiffness is a driving force causing hHpSCs to become hepatoblasts [37] and increasing rigid microenvironments eliciting differential maturation towards cholangiocytes [26, 37].

The mechanical forces can be altered in diseased states. Liver injury in cirrhosis is followed by expansion of hepatic stellate cells associated secondarily by altered matrix composition and extent of rigidity; this can result in portal hypertension resulting in apoptotic responses of parenchymal cells, phenomena that are paralleled by the responses to intraductal pressure manipulation via bile duct ligation [85, 86].

The cytoskeleton is a cellular component that fulfills the characteristics of amplification systems, in which all mechano-transduction mechanisms involving the cytoskeleton induce rearrangements of this network through modulation of Rho- and ROCK-associated proteins. The connections between the matrix and cells allow them to sense the rigidity of their environment through non-muscle myosin II, which directs stiffness-dependent differentiation in mesenchymal stem cells [75]. Germ layer organization and cell sorting depends on cell adhesion forces and cortex tension, which rely on actomyosin network activity [87]. Mechanical stretch in liver cells can induce the activation and synthesis of morphogens, such as those in the TGF- β family of Activin/Nodal signaling [88]. Integrins, a large family of transmembrane proteins involved in mechanical sensing, connect the cell to matrix substrates, recruit focal adhesions to adapt to mechanical stresses, bind external ligands and regulate intracellular signaling [83]. In TGF- β signaling, the transcription factor Smad2 is activated and reaches the nucleus through kinesin-mediated shuttling along intact microtubular networks [89]. In general, SMAD transcription factors are the backbone of TGF- β signaling pathways and fall into two categories: the receptor-mediated Smads (R-Smads) that work as substrates to activated cell receptors to transduce their signals; and common partnering Smads that either form complexes with R-Smads to sequester them and inhibit signaling cascades or shuttle to the nucleus and interact with other transcription factors to regulate gene expression [90].

Many cells in soft organs possess functional organelles like primary cilia that extrude microtubular structures into luminal spaces to detect flow effects at distances above cell apical surfaces and, as a result, amplify the effects of flow. Amplification of mechanical and chemical stimuli in primary cilia is possible through a polarized signal transduction pathway,

and uses the cytoskeleton to ensure faithful trafficking to the nucleus in a specific and non-diffusible fashion and trigger a global cellular response [91]. PDGFR α and Hedgehog signaling pathways take place in primary cilia [92, 93]. Hedgehog signaling pathways are present in liver of all donor ages [12], and involves dynein-mediated shuttling of Gli transcription factors [94]. Some noteworthy target genes of Gli transcription factors include PTCH genes, which introduce auto-regulation to the Hh signaling, as well as members of the TGF- β family like WNT and BMP genes, all involved in multiple embryonic development and differentiation mechanisms by their own function and by induction of other pathways [95-97]. Therefore, primary cilia and fluid flow are relevant participants in endoderm maturation and fate specification since Hh expression gradients demarcate the extension of endodermal organs during development [93, 98].

Mechanical interpretation of signaling mechanisms involved in liver development links differentiation with homeostatic control in adult stages. Future translational medicine strategies using stem cell biology principles will depend on biochemical signaling in appropriate physical environments, thus underlining the role of mechanics in tissue development.

Feedback Loop Signals and forms of Liver Regeneration

Signals produced by late lineage stage cells include both positive and negative regulators, and include bile salts, various soluble factors and components of the extracellular matrix [7, 74, 99, 100]. Bile secretion is an especially important mechanism of homeostatic control of tissue mass, operating as a mechano-transduction inductor. It is well known that

changes in bile tonicity also affect its viscosity and mechanical properties in a salt concentration-dependent fashion [101, 102]. Bile is reportedly a Newtonian fluid in normal physiological conditions whose tonicity increases down the biliary tree as hepatic parenchyma perform their secretory functions. Remarkably, abnormal bile tonicity is also characteristic of pathological conditions [103]. Since shear flow forces are proportional to viscosity, bile could function as a long-range mechanical signal that communicates to cholangiocytes in the proximal biliary tree the state of hepatic function across the entire liver maturational gradient through primary cilia bending, triggering stress-induced Ca^{2+} and cAMP signaling cascades as well as receptor-mediated PDGR α and Hedgehog signaling. Primary cilia in the periportal zone are responsible for homeostatic control of the liver by sensing the physical and chemical properties of bile [104].

Another facet of regulation is mediated by acetylcholine. It stimulates proliferation of both stem/progenitor cells and cholangiocytes expressing the M3 acetylcholine receptor [6, 105, 106]. In the normal liver, late lineage stage hepatocytes that lack M3 receptors produce acetyl cholinesterase, which destroys acetylcholine in the stem cell niche, thus blocking the proliferation of progenitor cells and cholangiocytes. This also occurs after partial hepatectomy, since the feedback loop is still intact. In contrast, during conditions of pericentral hepatocyte damage, acetylcholine is free to induce ductular reaction and cholangiocyte progenitor cell expansion. The modulatory effects by acetylcholine on the proliferation of cells lining the canals of Hering are absent in the denervated transplanted liver. The number of progenitor cells and reactive ductular cells in the transplanted liver injured by hepatitis were significantly lower than in innervated matched controls [106]. This clinical observation was confirmed at the experimental level where vagotomy induces an

impaired regeneration of progenitor cells and ductal reaction, in galactosamine damaged rat liver cholangiocytes [6].

Positive regulators include hepatopoietin, released by dying zone 3 cells and shown to stimulate expansion of stem/progenitors (M. Roach and J. Hambor, Pfizer Pharmaceuticals, unpublished observations). Another mechanism involved in the feedback loop signals is linked with the interaction between portal fibroblasts and cholangiocytes in periportal areas, regulated by the expression of the ecto-nucleotidase, NTPDase2 [107]. Under normal conditions, expression of NTPDase2 by portal fibroblasts inhibits the activation, mediated by extracellular nucleotides, of basolateral P2Y receptors expressed by cholangiocytes. After experimental cholestasis, portal fibroblasts lose their expression of NTPDase2, therefore allowing activation of P2Y receptors by nucleotides and downstream events upregulating cholangiocyte proliferation. Finally, cholangiocytes display primary cilia in their apical pole, which could be of relevance for maintaining cholangiocytes in a quiescent state because, as recently proposed, cilia bending maintains intracellular Ca_2 $[Ca_2]_i$ at a threshold where cAMP, an intracellular modulator of proliferation, is inhibited [108].

Feedback loop signalling is particularly relevant to the known forms of liver regeneration: 1) that which occurs after partial hepatectomy and 2) that which occurs after toxic injury to zone 3 (and sometimes also zone 2) parenchymal cells.

After partial hepatectomy, many (perhaps most) of the feedback loop signals remain intact. There is a wave of DNA synthesis across the liver plates, but with only a portion of the cells undergoing cytokinesis [69]. This results in an increase in polyploidy yielding hypertrophic cells with a higher level of expression of late genes, but also with a higher frequency of cells transitioning into apoptosis. Loss of cells by apoptosis is accompanied by

cell replacement from the stem cell compartment via an increased speed in the kinetics of the lineage. The stem/progenitors generate more cells to progress through the lineage and with time (typically weeks) gradually restore the normal ploidy profile.

After toxic injury to zone 3 (and sometimes also to zone 2) cells, there is a cellular “vacuum” of the late lineage stage cells, and the feedback loop signals are lost or drastically altered. The result is that the periportal cells undergo rapid, hyperplastic growth (complete cell division) followed by differentiation to later lineage stages. These phenomena, the classic “oval cell response” in rodents and the “ductular reactions” seen in human massive hepatic necrosis (e.g. acetaminophen toxicity, acute hepatotropic viral infection) have long been recognized to involve extensive expansion of the stem/progenitor cell populations [23, 109]. Chronic injury to the liver, as occurs with certain viral infections (e.g. hepatitis B or C), repeated drug exposures, or radiation result in loss of late lineage stage cells, and elicits chronic regenerative responses. Chronic regeneration can lead to mutational events associated with malignant transformation.

The feedback loop signals are an explanation also for the behavior of liver cells in culture. The diploid subpopulations, including the stem/progenitor cells, do not grow if they are co-cultured with late lineage stage parenchymal cells or are provided conditioned medium from the zone 3 cells [33].

Relevance of Cell Sources and Feedback Loop Signals to Clinical Programs

Lineages in fetal tissues are skewed towards early stages, particularly the subpopulations of stem/progenitors, and have no fully mature diploid cells (lineage stages 5

and 6) or polyploid cells (stage 7). Those in adult tissues have cells representative of all lineage stages (including stem/progenitors) but are skewed towards later lineage stages. The speed of turnover of a given lineage has a base rate and a more rapid rate induced by injury processes. Donor ages dictate the relative proportions of cells at particular lineage stages, so are therefore essential in sourcing of cell populations for therapies.

Recognition of the feedback loop will have bearing for many clinical programs. Liver cell therapies for inborn errors of metabolism must use strategies that accommodate the intact feedback loop signaling present. The patients are often difficult to manage clinically, and livers are for the most part normal, except for the effects of the defective gene. The liver's feedback loop in these patients is intact, so large numbers of stem cells must be implanted. The missing function(s) due to the genetic condition must be constantly monitored. Although the immunological issues are evident, one may be able to modulate immunology with the use of hepatic stellate cells to avoid immuno-suppression.

By contrast, patients in liver failure (conditions involving a loss of feedback loop signals) can be transplanted with smaller numbers of cells since there will be strong pressure for the transplanted cells (along with endogenous early lineage stage cells) to expand quickly to reconstitute the liver mass. Concerns include sufficient engraftment of cells to overcome liver failure, the possible rejection of cells once they mature. Again, the use of hepatic stellate cells (and/or angioblasts) from the recipient should alleviate the immunological issues. Most importantly, it will be essential to convert to grafting methods to optimize transplantation of liver cells and to identify non-invasive methods of monitoring cells after transplantation [110, 111].

Liver Cancer Stem Cells

An old idea, now revisited by many investigators, is that cancers are transformed stem cells or early progenitors. The idea originated with the pioneering work of Van Potter in the 1960s. He proposed that hepatomas contain cells undergoing “blocked ontogeny” [112, 113]. The idea was more carefully elucidated as one generic for all types of cancers as neoplasms of mutated stem cells by Barry Pierce and Stewart Sell [114-116]. Many functions thought to be related to cancer (e.g. α -fetoprotein expression in liver cancers) are now realized to be perfectly normal functions of an expanded stem/progenitor cell population. Therefore, current efforts focus on comparing cancer cells to their normal stem cell counterparts in order to identify the changes in a specific stem/progenitor cell population that have given rise to the malignancy [117-119]. Therefore, cancer cells are from a lineage stage at which cell division is a dominant feature [112, 120]. Indeed, investigators have found that normal stem/progenitor cells are strikingly similar to tumor cells in their appearance, their gene expression, and their growth properties, and that specific tumors, especially specific tumor cell lines, can be mapped or identified as an expanded lineage stage [119, 121]. It indicates that existing tumor cell line model systems can be used to define properties of their normal stem cell counterparts, and that comparison of those tumors to the normal counterparts should be extraordinarily revealing about key aspects of the malignant transformation process [118]. The clinical use of stem cells may come with an increased risk of tumors depending on the donors (e.g. if there are undiagnosed tumor cells among the endogenous stem cells) and on the patient’s medical condition (e.g. severe immunosuppression).

Cancer of the liver, whether primary or metastatic, must be treated with recognition of the feedback loop mechanisms, and that tumor cells are probable transformants of a stem/progenitor subpopulation. Treatments of the cancer with drugs or radiation kill later lineage stages. If they target also the mutated lineage stage(s), then the treatment can be curative. If they fail to target specifically the tumor cell, the transformed stem/progenitor is able to expand rapidly in the absence or reduction in feedback loop regulation. Therefore, future cancer therapies should involve strategies to use the feedback loop signals and/or regulators of the paracrine signaling pathways unique to stem/progenitors. Such therapies should prove more effective and less damaging to the health of the patient. Partial hepatectomy, as often occurs with treatment for patients with liver cancers, is associated with a slow regenerative response, since the turnover of the liver is predicated on an increased kinetic rate associated with increased apoptosis of the residual liver mass [69].

Treatment of patients will improve with strategies recognizing lineage biology. If a patient's tumor can be mapped to a specific lineage stage, then treatment of that patient (e.g. with chemotherapies, radiation therapies, etc.) must be targeted to the lineage stage(s) of aberrant cells. If the treatment eliminates cells at a later lineage stage but not the stage with the aberrant cells, then the treatment will actually worsen the patient's prognosis, since the feedback loop regulation will be eliminated by the treatment's killing of late (mature) lineage stages and subsequent disinhibition of the mutated cells [118, 119].

Conclusions

The intrahepatic maturational lineages have been defined, beginning at the stem cell compartment, located periportal and progressing through the midacinar region and ending

pericentrally. The parenchymal cells, along with their mesenchymal cell partners, are governed by gradients of paracrine signals, including both sets of soluble factors and insoluble extracellular matrix components, and by specific mechanical forces. Feedback loop signals regulate the stem/progenitors, controlling liver mass and tissue regeneration. Understanding the lineage biology in the liver and its regulation offers new considerations for developing strategies tailoring potential therapies for patients in clinical programs.

CHAPTER II

The Future of Cell Transplant Therapies: A Need for Tissue Grafting

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Abstract

Current methodologies of solid-organ derived cell transplant therapies introduce donor cells into hosts via a vascular route, a strategy modeled after hematopoietic therapies. These strategies fail due to inefficient engraftment, poor survival of the cells, and propensity for formation of life-threatening emboli. Transplant success necessitates grafting methods, requiring a mixture of appropriate cell sources embedded into or onto precise mixes of extracellular matrix components and then localized to the diseased or dysfunctional tissue, promoting necessary proliferation, engraftment and vascularization. Grafting technologies are rapidly translatable to therapeutic uses in patients and provide alternative treatments for regenerative medicine.

Introduction

As the field of regenerative medicine continues to advance, transplant physicians around the world have launched or are about to launch clinical trials transplanting cells from solid organs into recipients to treat a number of diseases and conditions, including acute liver failure, diabetes, spinal cord injury, and myocardial infarctions. Although some conditions like diabetes have been treated with limited success by islet transplantation, the diseases of most solid organs have yet to be treated successfully. Cell transplantation involving solid organ-derived cells are typically performed via a vascular route, and results routinely show overwhelming evidence of inefficient engraftment and marginal effects. We propose that successful therapies involving cells from solid organs will be accomplished with strategic *grafting protocols*. Examples include skin grafting that is performed with existing methods and FDA approved reagents. The exact method of grafting can be precisely tailored to the patient's needs.

The era of cell transplant therapies first began with hematopoietic stem cells as therapies for cancer. Strategies pioneered by E. Donnall Thomas, began with human bone-marrow grafting into patients after total body irradiation, resulting in the recovery of bone marrow and lymphoid tissue [122]. Hematopoietic cell therapies are easily performed as these cells are maintained in suspension and have inherent features, including the acquisition of antigens, which support their homing to specific target tissues. However, the studies involving transplantation of hematopoietic cell subpopulations have little relevance to applications from solid organs such as skin or internal organs (e.g. liver, lung, heart). Results from the initial clinical trials involving cell therapies have been encouraging [123-125], but the results are compromised by inefficient engraftment, lack of vascularization, lack of

growth when transplanting mature cells, and highly immunogenic properties of the cells necessitating long-term immunosuppression. Other hurdles include the sourcing of clinical grade, high-quality cells and the need to use freshly isolated cells due to difficulties with cryopreservation [126].

Engraftment efficiencies for cell transplants remain low, since many cells die during the engraftment process or become located in organs other than the target tissue [127-130]. For example, cardiomyocytes injected into the heart after injury are washed out via the vasculature into other organs in the body [131]. Similarly, injection of liver cells via vascular routes results in donor cells in most if not all of the tissues of the body [132]. In addition to the inefficiencies, cell transplantation via a vascular route is potentially lethal. The cells from solid organs have surface molecules (cell adhesion molecules, tight junction proteins) that make the cells bind to each other rapidly and enhance aggregation. This clumping phenomenon can result in life-threatening pulmonary emboli.

Different strategies are required for cells from solid organs. The development of grafting technologies involve the delivery of transplanted cells as an aggregate on or in scaffolds that can be localized to the diseased tissue to promote necessary proliferation and engraftment. Thus, one must look not only at the cell type to be transplanted, but the cell type in combination with the appropriate biomaterials and grafting method for the most efficient and successful transplant therapies.

Cell Sourcing and Requisite Cells

Cell sourcing for different therapies requires the use of lineage-staged populations. Progenitor cells establish all lineage stages of the tissue. However, the use of later lineage stages are preferred if there is a need for rapid acquisition of functions offered only by the late lineage cells, or if the recipient has a lineage-dependent virus that preferentially infects the stem cells and/or progenitors such as what occurs with hepatitis C [133] or papilloma virus [134].

The graft's cellular components should mimic the categories comprising the epithelial-mesenchymal cell relationships, the cellular foundation of all tissues. For stem cell grafts, the cellular populations comprise angioblasts, the epithelial stem cells, and “nurse” cells, providing critical regulatory signals, both soluble and insoluble extracellular matrix components. Using liver cell therapies as a model a stem cell graft comprises the hepatic stem cells, angioblasts and hepatic stellate cell precursors [19]. In contrast, a mature liver graft comprises hepatocytes, mature endothelial cells and pericytes, the mature stellate cells [135, 136].

The ideal ratios of the cell populations should replicate those found *in vivo* and in cell suspensions of the tissue. The mix of cells allows for maturation or maintenance of the adult cell types along with requisite vascularization. The issue of vascularization is extremely important for all grafts, as shown by the initial frustrations with pancreatic islet transplants. Success was achieved with the Edmonton protocol, in which cells were transplanted into the liver, a site providing the needed vascularization, as well as the use of specific drugs to control immunological reactions. For most conditions, we assume that stem cell grafts will be ideal, given their expansion potential, their ability to mature into all of the adult cell types,

their tolerance for ischemia, enabling their sourcing from cadaveric tissue, and their minimal immunogenicity [137-139].

Grafting Materials

Successful grafting strategies can be guided by clinical programs involving skin. As an alternative to skin autografts or allografts, artificial skin is created using biomaterials. This approach provides a scaffold for cell support and signaling that assist in the success of the grafting and regenerative processes. As living tissue in an organism undergoes constant remodeling, dissociated cells tend to reform their native structures under appropriate environmental conditions. Normal epithelial cells from solid organs are anchorage dependent and require a matrix scaffold, various soluble signals, and a three dimensional (3-D) structure to guide regeneration [140].

Synergistic effects between soluble and matrix factors dictate growth and differentiative responses by the cells [141-143]. The matrix components are the primary determinants of attachment, survival, cell shape (as well as the organization of the cytoskeleton), and stabilization of requisite cell surface receptors that prime the cells for responses to specific extracellular signals [33].

The choice of matrix components includes selection of particular collagens, adhesion molecules, proteoglycans (PGs) and their glycosaminoglycan (GAG) chains. There are at least 25 collagen types, each one encoded by distinct genes and with unique regulation and functions [144, 145]. In parallel, there are multiple families of each of the other categories of matrix components [74, 146]. Alternative biomaterials can also be considered. Inorganic,

natural materials like chitosan and alginate are promising materials, as are many synthetic, biodegradable and biocompatible polymers [147-149]. These materials are often solidified through methods including thermal gelation, photo cross-linking, or chemical cross-linking. With each method it will be important to use strategies that account for potential cellular damage (e.g. temperature ranges, UV exposure).

Fortunately, the selection of which matrix components to use is guided by gradients *in vivo*, that change from the stem cell compartment to the late lineage stage cells. The chemistry of the matrix molecules changes with maturational stages, with host age, and with disease states [150]. Therefore, graft biomaterials should mimic the matrix chemistry of the particular lineage stages desired for the graft. One can test the efficacy of the mix of matrix components in *ex vivo* studies using purified matrix components and soluble signals, many of which are commercially available, many in GFP forms, and can be used in clinical trials [151, 152]. The biomaterials selected for the graft should elicit the appropriate growth and differentiation responses required for successful cell transplantation. Grafting with appropriate materials should optimize engraftment of transplanted cells in a tissue, prevent dispersal of the cells to ectopic sites, minimize embolization problems, and enhance the ability of the cells to integrate within the tissue as rapidly as possible. Moreover, the factors within the graft can also be chosen to minimize immunogenicity problems.

Grafting Methods

Grafting methods will eventually become a major field for clinical and experimental research. The type of grafting material also has an influence on the methodology as some methods enhance localization to certain tissue types, as seen in **Figure 6**. For tissues where

grafts would replace a diseased or missing tissue (bone, for example), an implantable solid matrix allows cells to be seeded with necessary growth factors into the matrix, cultured, and then implanted into the patient.

Injectable grafts are likely to dominate future cell therapies, as these grafts can fill any deficit shape or space (e.g. damaged organs or tissues). Co-culturing and injecting with a cell suspension embedded in gelable biomaterials allow for solidification *in situ*, using the various crosslinking methods available for a given biomaterial chemistry, and can easily incorporate soluble signals or specific matrix components. Since these grafts can easily be injected into the target area, there is no or minimal need for invasive surgery, reducing patient discomfort, lowering risk of infection, scar formation, and keeping down overall cost [153]. In some cases, one can design a graft to be placed onto the tissue's surface, in which case the graft would be held in place with a biocompatible and biodegradable covering. For some abdominal organs, this covering could be from autologous tissues such as a patient's omentum.

The ability of the transplanted cells to survive and expand in the recipient is affected by whether the tissue in the recipient is in a quiescent or regenerative state. Patients with acute or chronic liver failure suffer from a loss of late lineage stage cells (i.e. pericentral parenchymal cells). These late lineage stage cells are the source of feedback loop signals that regulate the kinetics of the liver turnover. With liver failure, there is a cellular "vacuum" and muting or loss of feedback loop signals, resulting in rapid expansion of both the transplanted cells and host periportal cells, followed secondarily by differentiation to later lineage stages [109]. Patients in liver failure can be transplanted with smaller numbers of cells per body weight of the patient, since there will be strong pressure for transplanted cells to expand

quickly and join with the endogenous periportal cells to reconstitute the liver mass. The expansion of cells is increased when combined with hepatic irradiation [154, 155]. It is estimated that 1-5% of the liver mass is needed for regeneration therapies, corresponding to 8.8×10^9 hepatocytes for a human liver.

If transplanted into quiescent tissue, as occurs in patients with inborn error(s) of metabolism, the donor cells engraft and minimally expand. Late lineage stage cells are present and producing the feedback loop signals that inhibit or slow the kinetics of the liver lineage. Liver cell therapies for patients with inborn errors of metabolism require relatively large numbers of cells, or multiple treatments, to compensate for the minimal expansion potential of the transplanted cells.

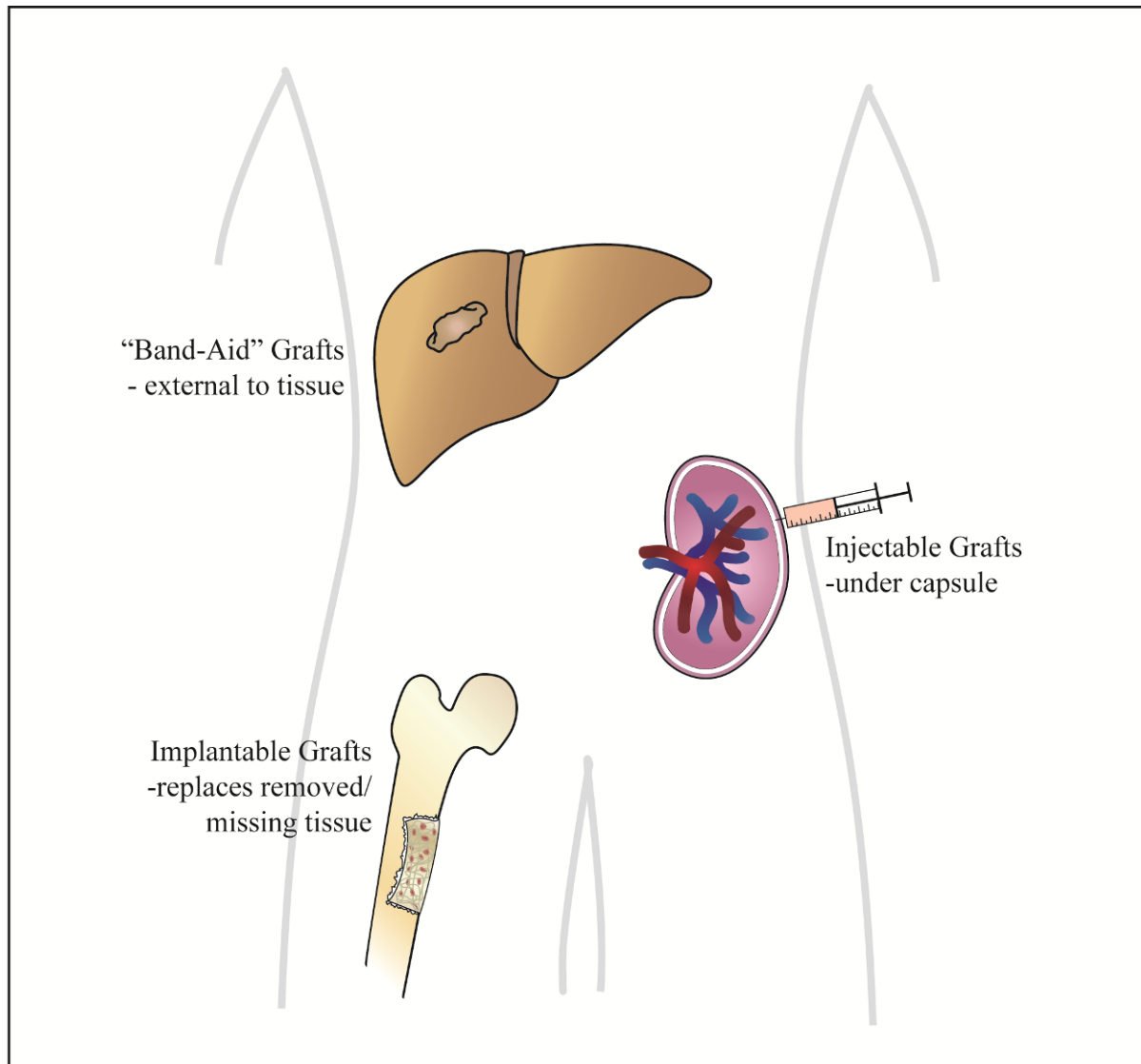


Figure 6: Methods for grafting to different target tissues, including implantable grafts, injectable grafts, and patch grafts. Dependent on the diseased tissue type, one method may be more appropriate than another.

Conclusions

In tissue engineering, the key goal is to mimic the complexity of the native microenvironment with a minimum number of components that allow transplantation of cells to successfully engraft, expand and then rebuild the tissue. At the same time, a one-combination-fits-all solution is not logical. Different tissue niches require different strategies

to allow for maximum regeneration, and the grafts must be specifically designed for tissues. By combining the native epithelial-mesenchymal partners of a specific maturational lineage stage in combination with the lineage-specific biomaterials (the matrix components and soluble signals), cells will be in a specific microenvironment needed for optimal regenerative response. Grafting should be localized to the diseased or dysfunctional tissue, minimizing the loss of cells to ectopic sites. The use of grafts can facilitate cell transplantation, greatly reduces the cost for the procedures, and allow for scalable grafts, enabling transplantation of large numbers of cells. Technologies developed are rapidly translatable to therapeutic uses, and can benefit patients by providing an alternative treatment to transplants that may regenerate lost tissue.

CHAPTER IV

Cryopreservation of Human Hepatic Stem/Progenitor Cells- Effects of Hyaluronans on the Cells and their Adhesion Molecules

Rachael Turner, Gemma Mendel, Elaine Wauthier, and Lola M Reid

Abstract

The supply of human hepatic cells (hHpSCs) is often limited and dependent on the availability of liver tissues from surgical resections and rejected organs from organ donation programs. To optimize this scarce resource, an effective method is needed for cryopreservation of cells, yielding consistent ability for the cells to survive freezing and to grow and function in culture after thawing for use in both research and clinical applications. The aim of this study was to assess the effects of hyaluronans (HA) to improve preservation of adhesion mechanisms that could facilitate culturing the cells and preservation of functions postthawing. Freshly isolated hHpSCs and hepatoblasts (hHBs) were isolated from fetal livers and cryopreserved in one of a number of different cryopreservation buffers, with or without supplementation of 0.5 or 0.10% hyaluronans (HA). All of the buffers tested yielded high viabilities (80-90%) on thawing. However, supplementation with HA showed considerable improvement in the ability to attach and to be cultured. Best results observed were for cells cryopreserved in CS10 isotonic medium supplemented with small amounts of hyaluronans (0.05 or 0.10%). Success correlated with improved expression of adhesion

molecules (Integrin β 1, E-Cadherin). The findings reveal improved methods in cryopreservation of freshly isolated human hepatic progenitors under serum-free conditions, offering more efficient methods for stem cell banking in both research and potential therapy applications.

Introduction

On a cellular level, the start of hypoxemia is initiated the moment a tissue is removed from a living organism; it leads quickly to tissue and organ damage. Freezing cells is a method to arrest cells for future use and leads to the continuing interest on understanding the ability of cells to survive freezing to be used in numerous therapeutic possibilities. Due to the common sourcing issues with human cells, extensive research has been done on methods of cryopreservation and viability and proliferation after thawing. If proliferative cells can be successfully cryopreserved and recovered, many sourcing problems in clinical therapies can be alleviated. Cryopreservation of hepatocytes is extremely difficult due to the sensitivity of liver cells, causing them to be easily damaged by the freezing and thawing processes [156]. In addition, stem and progenitor cells have proven difficult because they are more likely to lineage restrict during cryopreservation, caused by the freezing process and/or the added factors [156]. Unprotected freezing is normally lethal, and standard buffers and procedures have been adopted beginning as early as 1949 with Polge, Smith and Parke's paper preserving spermatozoa to survive prolonged freezing at -80°C using 10-20% glycerol [157]. Several factors have been investigated when studying cryopreservation techniques, including the use of cryopreservation agents [158, 159], encapsulation techniques [160-162], pre- and post- treatment of cells [163], and regulation of freezing temperature [164, 165]. Current challenges to hepatic cell cryopreservation are the loss of cells, cell viability (at best 62-85%) and high variability (5-95%) in cell adherence to culture dishes in post-thawing [163, 166, 167]. Some of the key variables identified are summarized below:

Isotonic Media. The usual culture media are not isotonic and, upon freezing, result in massive influx of water into cells, that in turn results in ice crystal formation and damage or death to the cells. This is prevented by the use of isotonic media prevents and greatly improves cell survival, ability to attach and grow in culture and differentiated functions [168-172].

Cryopreservation agents. The function of cryoprotectants is to avoid ice formation and cellular dehydration [159]. Common methods of cryopreservation include the use of a cryopreservation agent to reduce internal ice formation, and include dimethyl sulfoxide (DMSO), glycerol, trehalose, ethanediol, and propanediol that permeate the cell, and sugars and polymers that are non-permeating such as PVP dextrans, [158, 159, 163, 173]. Glycerol is found in the cells and tissues of many organisms living in freezing conditions including the Artic, Antarctic and high mountains [174], but its viscosity makes it difficult to utilize in routine procedures. Dimethyl sulfoxide (DMSO) at 2-20% is commonly used for freshly isolated (primary) cells. DMSO is more soluble than glycerol in aqueous buffers and penetrates cells readily making it the most commonly used cryopreservation agent. The only concern for use of DMSO is that it has been reported to drive differentiation of hemopoietic stem cells [175]. Rate of DMSO addition is important, as DMSO in cell suspension causes osmolarity of the solution to be many times higher than solutions without it. Therefore, the slow addition of this cryoprotectant may be beneficial for the cells to allow them to adjust to the change of osmolarity [176]. It was found important to remove the DMSO quickly after thawing to avoid damage to the cells by prolonged exposure to DMSO [177].

Antifreeze Proteins. (AFPs) or ice structuring proteins (ISPs) facilitate cell survival under freezing conditions. They were first identified in fish found in Artic or Antarctic [178]

but have been found in the cells of many species that live in such frigid environments. They involve mechanisms that include interactions with cell membranes that protects the cells and in the establishment of thermal hysteresis, a process that results in the inhibition of growth of ice crystals.

Antioxidants. A combination of antioxidant compounds can treat the stress of isolation that occurred when hepatocytes were taken from the liver prior to cryopreservation [179]. The hydroxyl radicals, lipid peroxides, nitric oxide and hydrogen peroxides in the isolated cells were scavenged by the antioxidants to prevent cell damaging. Addition of antioxidants to the cryopreservation buffers results in increased cell survival and preservation of cell functions after freezing.

Regulation of rate of freezing process. Mazur first published in 1963 the finding that the rate of temperature change plays a large role in cell viability during cryopreservation because the rate of change of temperature affects the transport of water and solute across the cell membrane [173, 180]. Today, it is accepted that mammalian cells can be cooled and stored in liquid nitrogen temperatures by freezing with a cryoprotectant at a rate of about -1°C/min by placing cells in a freezing container at -80°C for a few hours and then moving to liquid nitrogen storage (below -130°C); this method is better known as progressive freezing (PF). In stepwise freezing (SF), the cells are brought down in steps of set time intervals with controlled temperature rates in a controlled rate freezer [167].

Studies on Liver Cells

There are many publications on the cryopreservation of mature hepatocytes, and more recently focus on cryopreservation of stem cells with their ability to proliferate and function after freezing. Rat small hepatocytes, diploid cells that are ~17-18 μm diameter, were maintained at -80°C for over 6 months, and 60% attachment and proliferation of colonies was observed [156]. Liver function including albumin production and other serum proteins was also observed. Studies on the cryopreservation of adult human hepatocytes obtained from liver biopsies compared differences in freezing methods, including stepwise freezing (SF) and progressive freezing (PF) [167], both previously developed methods [181]. Freshly isolated hepatocytes showed a viability of 88% and plating efficiency of 79%. After cryopreservation/thawing using SF, only 12% recovery was seen. PF gave higher recoveries of 38%. Of this 38%, 40% of the viable cells were shown to successfully plate. When the PF samples were pre-incubated in medium for 30 minutes, the plating efficiency increased to 64%. Conclusions involving the cryopreservation of human hepatocytes show greatest success with a progressive freezing following a pre-incubation and could potentially be used in cell banking.

Work has also been done involving the cryopreservation of hepatic stellate cells [182], which as discussed previously are an important player in the successful culturing of hHpSCs and their descendents. Both cooling rates and cryoprotectants were investigated. It was found that DMSO as a cryoprotectant gave superior attachment and viability. Recent studies that freeze cells in media designed to be isotonic at freezing temperatures, instead of culture media, have shown to improve primary cells and stem cell survival [168-172].

The non-attachment of cryopreserved hepatocytes may in large part be due to the loss of cell adhesion molecules, specifically β 1-integrin and E-cadherin molecules. Both proteins have been found to be significantly reduced after cryopreservation [183]. Freezing cells within hyaluronans may inhibit this protein degradation, allowing for better cell attachment. In addition, studies on the entrapment of hepatocytes within alginate beads has been shown to have protective effects [184]; the cells within the alginate beads can be transplanted into rats after freezing, still maintaining their viability [185]. In addition, a hyaluronan matrix can provide a facile way to transfer cells from one condition to another. Given the presence of hyaluronans in the native stem cell niche and the protective properties seen in other entrapment methods, freezing using hydrogels may prove to be an optimal method for increased cell viability and attachment, to be used for future therapeutic strategies.

In this study, we evaluated cryopreservation conditions, including different isotonic mediums with and without the presence of hyaluronans, to determine the best freezing method for future cell banking. Cell viability, expression of cell adhesion molecules, and immunohistochemistry on matrix components were used to assess and determine the optimal freezing conditions of human hepatic progenitor cells.

Methods

Liver sourcing and processing

Liver tissue was provided by an accredited agency (Advanced Biological Resources) from fetuses between 14-20 wk gestational age that were obtained by elective terminations of pregnancy. The research protocol was reviewed and approved by the Institutional Review

Board for Human Research Studies at the University of North Carolina. All processing and cell enrichment procedures were conducted in a cell wash buffer composed of a basal medium (RPMI 1640) supplemented with 0.1% bovine serum albumin, BSA (BSA Fraction V; Sigma-Aldrich), insulin and iron-saturated transferrin (both at 5 ug/ml; Sigma-Aldrich), trace elements (300 pM selenious acid and 50 pM ZnSO₄), and antibiotics (AAS; Invitrogen). Liver tissue was subdivided into 3-ml fragments (total volume ranged from 2 – 12 ml) for digestion in 25 ml of cell wash buffer containing type IV collagenase and deoxyribonuclease (both at 6 mg per ml; Sigma- Aldrich) at 32°C with frequent agitation for 15 – 20 min. This resulted in a homogeneous suspension of cell aggregates that were passed through a 40-gauge mesh and spun at 1,200 RPM for 5 min before resuspension in cell wash solution. Erythrocytes were eliminated by either slow-speed centrifugation or by treating suspensions with anti-human red blood cell antibodies (1:5,000 dilution; Rockland) for 15 min, followed by LowTox Guinea Pig complement (1:3,000 dilution; Cedarlane Labs) for 10 min, both at 37°C. Estimated cell viability by Trypan blue exclusion was routinely >95%.

Hepatic Stem Cell Culture Conditions

The fetal human liver cells were suspended into a serum-free medium tailored for hepatic progenitors consisting of a serum-free basal medium (RPMI 1640, Gibco–Invitrogen, Carlesbad, CA) containing no copper, low calcium (< 0.5 mM) and supplemented with insulin (5 ug/mL), transferrin/Fe (5 ug/mL), high density lipoprotein (10 ug/mL), selenium (10M), zinc (10M) and 7.6 uE of a mixture of free fatty acids bound to purified albumin; the detailed methods for its preparation have been published elsewhere[33].

Fresh fetal liver cell mix was plated at ~300,000 cells per 100mm² round culture dish (78.5cm² surface area) or ~ 500,000 cells per plate in a standard 6-well (10cm² surface area per well). The low cell concentration and the conditions in KM were not conducive to survival of mature liver cells. If serum is added, the angioblasts lineage restricted rapidly to the hHpSTC precursors and to mature hHpSTCs that subsequently dominated the cultures. Use of serum-free conditions was essential to keep the hHpSCs and angioblasts stably in the lineage stage enabling them to self-replicate. Cells were plated with KM with 10% fetal bovine serum (FBS) for at least 6 hrs and up to 24 hrs to facilitate attachment but transferred under serum-free conditions thereafter. Cell media were changed every 3-4 days with serum free KM. Typical plates had single cells and small clusters of cell that adhered after the initial 24hrs. Colonies began to appear after 1-2 weeks.

Freezing Conditions

Freshly isolated human hepatic progenitors were allowed to recover at 4°C for 2 hrs prior to freezing in KM. Samples were frozen at 2E6 cells /1ml in cryopreservation vials (Nunc). Cryopreservation solution consisted of either culture medium supplemented with 10% DMSO or CryoStorTM-CS10 (Biolife Solutions), and with 0, 0.05, or 0.10% Hyaluronan hydrogel (Glycosan) by weight. Cells were allowed to equilibrate in the cryopreservation solution for 10 min at 4°C.

Samples were frozen in a low percent of hyaluronan (HA) that was uncrosslinked. In general the hyaluronans increase the sample viscosity, but HA without cross-linker does not form a rigid gel. Samples were prepared by mixing three parts: cells in freezing medium, gel in freezing medium, and freezing medium to adjust volume. Hyaluronan from HyStem Kit

(Glycosan), comes in sterile bottles dissolved by addition of 500 μ L DI water and 15minutes in warm water bath 37°C, making a 2% HA solution. Then 200 μ L of DMSO and 1300 μ L of culture freezing media (KM, CryoStor™-CS10) were added to make a 0.5% gel in either media. A summary of freezing and thawing methods, with temperatures, can be seen in **Figure 7**.

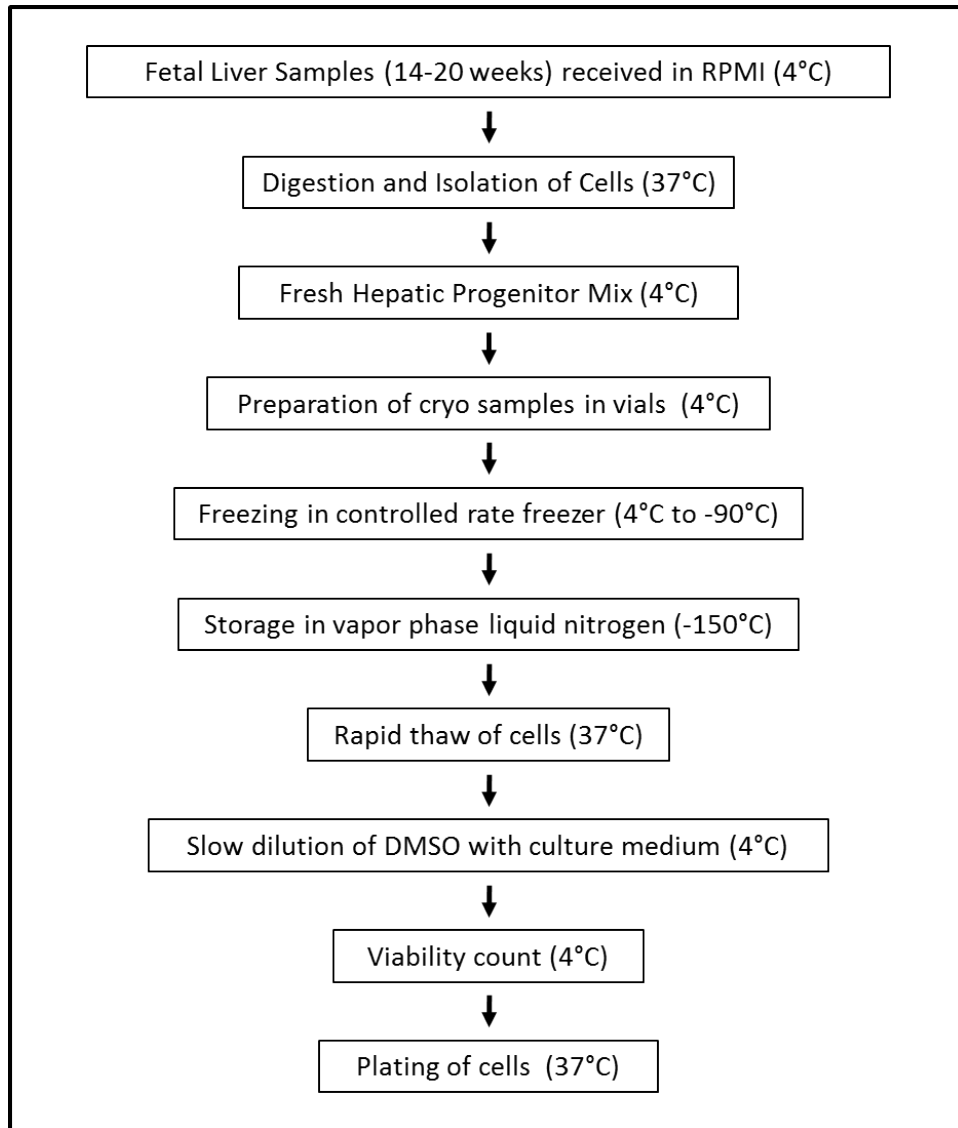


Figure 7: Schematic of cryopreservation and thawing methods.

Freezing Rate

A controlled freezing rate program was devised to provide a repeatable program for freezing cryo vials that maximized cell survival. Samples were frozen using a Cryomed 1010 control rate freezer (Forma Scientific). The freezing program was modified after Hubel et al [186], and can be seen with the chamber and sample temperatures with respect to time in

Figure 8.

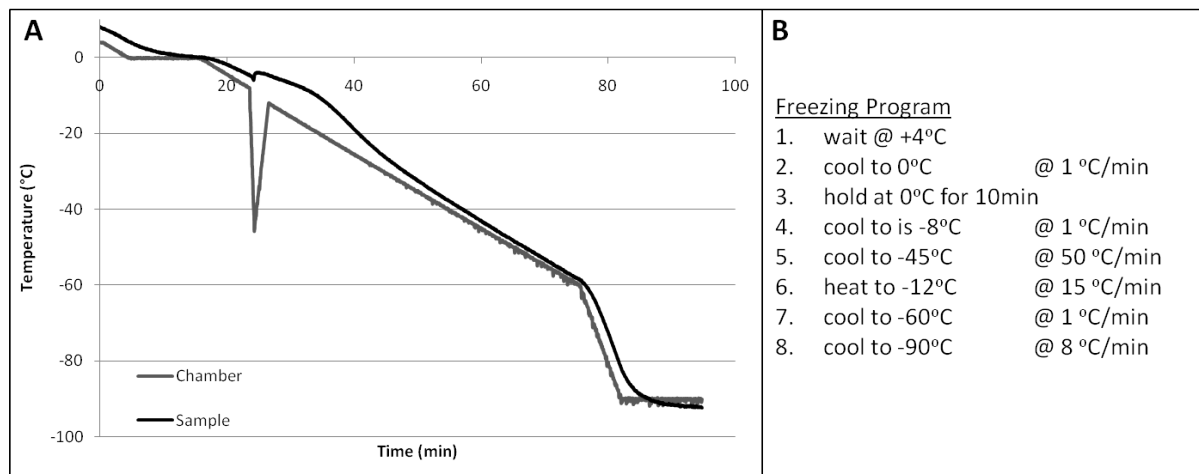


Figure 8: Controlled rate freezing program minimizes liquid-ice phase entropy preventing internal ice damage and allows for repeatable freezing. A) Graph shows chamber temperature in relation to sample temperature (10% DMSO). B) freezing program rates used for Cryomed 1010 system.

Plating on Collagen III coated plates

Cells were plated onto 6-well cell culture plates (Becton-Dickinson and NUNC) that provide a charge to the polystyrene facilitating cell attachment. It is known that cryopreservation with DMSO causes a decrease in adhesion molecules and attachment, either mechanically or through down regulation of gene expression [182, 183]. Plates were coated

with collagen III at 1 $\mu\text{g}/\text{cm}^2$ (Sigma-Aldrich, type X) to facilitate stem cell attachment by more closely resembling the normal microenvironment of the stem cells [25].

Viability

Cell viability was determined post-thaw by Trypan blue exclusion assays. Samples were thawed by quickly heating vials in a warm water bath at 37°C until the ice is melted. Samples were slowly diluted with culture media at room temperature or on ice at approximately 1 ml/min. The hepatic cell mix was diluted to <0.5% DMSO when plated. After cell counting, the cells were plated in culture media on 6-well plates, containing a 9.8 cm^2 attachment area per well, at 500,000cells/plate.

Colony Counting

Hepatic progenitor colonies began to appear between 1-2 weeks after plating, and the tight cells cluster were observed by looking at the plate with the naked eye, but easily were identified by inspection at 10X with a light microscope. Any size colony was counted as one, whether large ones at >3000 cells or small ones at <200 cells. Each well of 6-wells was looked at using 10X magnification for colonies and counted after 2-3 weeks of culture. Observations of colony number, size and morphology were noted.

Quantitative Real Time PCR (qRT-PCR).

Gene-specific primer sequences for quantitative gene expression analysis of differentiation markers [17, 33, 189, 190] were designed and validated for PCR doubling

efficiency and priming specificity using cDNA templates assembled from Total RNA of relevant cell lines (listed in Table 5) positively expressing each gene of interest extracted with the RNeasy Mini Plus kit (QIAGEN). Quantitative Real Time PCR (qRT-PCR) measurements were performed with an Applied Biosystems® 7500 Real-Time PCR System available in the Functional Genomics Core Facility at UNC Chapel Hill. Primers used can be found in Table 4. All measurements of relative expression were normalized with respect to GAPDH by the absolute quantification method described elsewhere [191, 192].

Table 4: Primer sequences used for quantitative RT-PCR.

Gene	Description	Primer Sequence (5' → 3')	NCBI Ref. Seq.
CDH1	E-cadherin	Forward: TCACAGTCACTGACACCAACGA	NM_004360.3
		Reverse: GGCACCTGACCCTTGTACGT	
		Reverse: GCCAGCTTTGAGCAAATGACAGTATTTTG	
CD44	Hyaluronic acid receptor	Forward: TGCCGCTTTGCAGGTGTAT	NM_000610.3
		Reverse: GGCCTCCGTCGAGAGA	
ITGB1	Integrin β 1	Forward: CAAAGGAACAGCAGAGAAGC	NM_002211.3
		Reverse: ATTGAGTAAGACAGGTCCATAAGG	
ITGB4	Integrin β 4	Forward: CTGTGTGCACGAGGGACATT	NM_000213.3
		Reverse: AAGGCTGACTCGGTGGAGAA	
		Reverse: CTCCGGAGGCTTCACAGGTA	
GAPDH	Glycerine aldehyde-3-phosphate dehydrogenase	Forward: AAGGTGAAGGTCGGAGTCAA	NM_002046.3
		Reverse: AATGAAGGGTCATTGATGG	

Immunohistochemistry.

By 24 hrs after plating, cells were fixed for 2 hrs with 4% PFA. Plates were then washed with PBS, bordered using a PAP-PEN, and blocked for using PBS + 0.1% Triton + 10% goat serum. Primary antibodies were applied and allowed to incubate, followed by another wash and the application of secondary (Alexa Fluor) antibodies. Once tagged, a mounting medium containing DAPI was applied and slides were imaged using confocal

fluorescent microscopy. For a list of primary and secondary controls, please see **Table 5**. Positive control cell lines are listed, and samples stained with only secondary antibodies will be used as negative controls.

Table 5: Antibodies and respective positive control cell lines used for immunohistochemistry.

Marker	Description	Manufacturer (Cat. No.)	Source (Isotype/Emission)	Stock Concentration	Titer	Positive (Cell Line)
CDH1 (human)	E-cadherin	Abcam® Inc. (ab8993)	mouse anti-human (IgG _{2B})	1 mg/ml	1:100	Hep3B
EPCAM (human)	Epithelial cell adhesion molecule	Lab Vision/Neomarkers (MS-181-P1)	mouse anti-human (IgG ₁)	200 µg/ml	1:500	
CD44 (human)	Hyaluronan receptor	Abcam® Inc. (ab6124)	mouse anti-human (IgG _{2A})	100 µg/ml	1:100	MDA-MB-231
IgG _{2A} (mouse)	Alexa Fluor® 647	Invitrogen™ Molecular Probes® (A690311)	goat anti-mouse (647 nm)	2 mg/ml	1:800	N/A
IgG _{2B} (mouse)	Alexa Fluor® 488	Invitrogen™ Molecular Probes® (A21141)	goat anti-mouse (488 nm)			
IgG ₁ (mouse)	Alexa Fluor® 568	Invitrogen™ Molecular Probes® (A21124)	goat anti-mouse (568 nm)			

Microscopy

Cells stained and tagged with secondary antibody fluoroprobes were identified using fluorescent microscopy. Negative controls were used to normalize backgrounds and eliminate image acquisition noise. Microscopy was performed at the Microscopy Core Laboratory, a CORE laboratory of UNC-CH. An Olympus IX 81-ZDC Inverted Fluorescence Microscope was used, with Phase Contrast, and fluorescent filters at 350nm, 488nm, 568nm, and 647nm. Equipped with a fully motorized stage, computer controlled by Improvion's Velocity software, and a Hamatsu ORCA RC camera and QImaging RETIGA 4000R color camera.

Statistical analysis

Results were presented as mean \pm standard error of the mean. Statistical analysis of data was performed by a one-way ANOVA. Significant findings were followed with pair-wise t-tests corrected for multiple comparisons using the step-down Bonferroni method.

Results

Effect of freezing conditions and hyaluronans on cell viability and colony counts

Immediately after cell thawing, the viability of thawed cells was assessed. After viability was determined, cells were seeded at equal densities on collagen III coated plates, as discussed in the methods, and allowed to grow. At 2-3 weeks, stem cell colony formation was assessed to determine the ability to attach and form colonies. The viability and cell colony formation counts for the various freezing conditions are summarized in **Figure 9**. Although there was a trend seen in viability post-thaw, where cells frozen in CS10 with hyaluronan hydrogels showed better viability percentages (KM= 83.2% \pm 1.2%, CS10+0.05%HA = 86.8% \pm 1.6%), there was no statistical difference between the samples. Similarly, no statistical significance was seen in the colony counts.

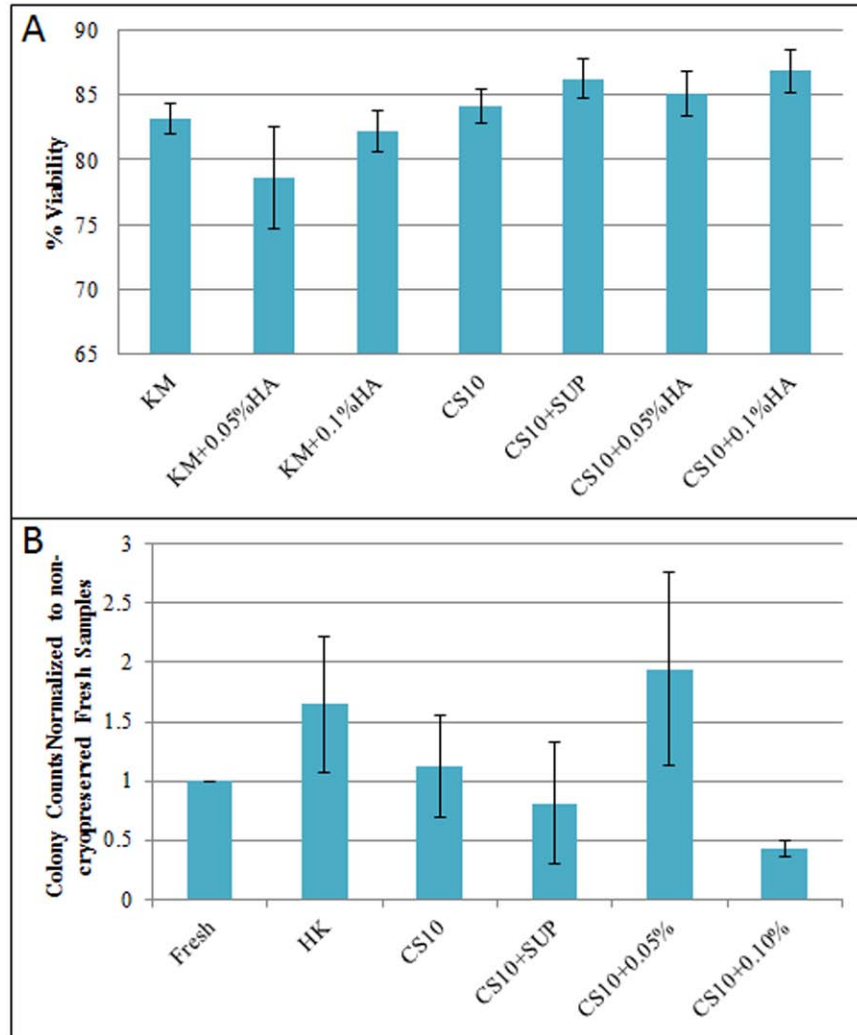


Figure 9: A) Cell Viability % of cryopreserved fetal hepatic cells post-thaw. B) Colony counts after 3 weeks of culture for each condition, normalized to fresh samples. Results are reported as mean \pm standard error of the mean. KM= Kubotas Medium with 10% DMSO and 10% FBS. CS10=cryostor, CS10+sup=cryostor10 with KM supplements. 0.05% and 0.10% refer to the HA% supplemented in each sample.

Effect of hyaluronans on genetic expression of cell adhesion molecules and immunohistochemistry of adhesion proteins.

A closer look was given to the expression of key cell-cell and cell-matrix adhesion factors. A summary of the genetic expression profiles of cell adhesion molecules in cryopreserved samples can be seen in **Figure 10**. The highest expression of Integrin β 1 post-

thaw was seen in samples frozen in CS10 +0.05% HA (0.130 ± 0.028 , $n=28$). This is significantly different when compared to expression seen in fresh samples (0.069 ± 0.007 , $n=24$, $p<0.01$). Integrin β 4 expression between fresh and cryopreserved samples was not seen to be significant ($p<0.05$). CDH-1 (Ecadherin) expression in cells frozen in CS10+0.1%HA (0.049 ± 0.006 , $n=20$) and CS10+0.05%HA (0.064 ± 0.003 , $n=16$) showed significant increases in expression when compared to fresh samples (0.037 ± 0.005 , $n=36$, $p<0.05$). Expression in CD44 between samples were also very similar, with negligible significant differences, though a trend of higher expression was seen in samples frozen with HA.

Immunohistochemistry on cells 24hrs after thaw in CS10 sample conditions showed positive co-staining for E-cadherin in Ep-CAM positive hepatic progenitors (hHpSCs and hHBs). When cells were frozen with HA, increased CD-44 stained feeder cells were more abundant near paranchymal cell colonies when compared to those samples without hyaluronans.

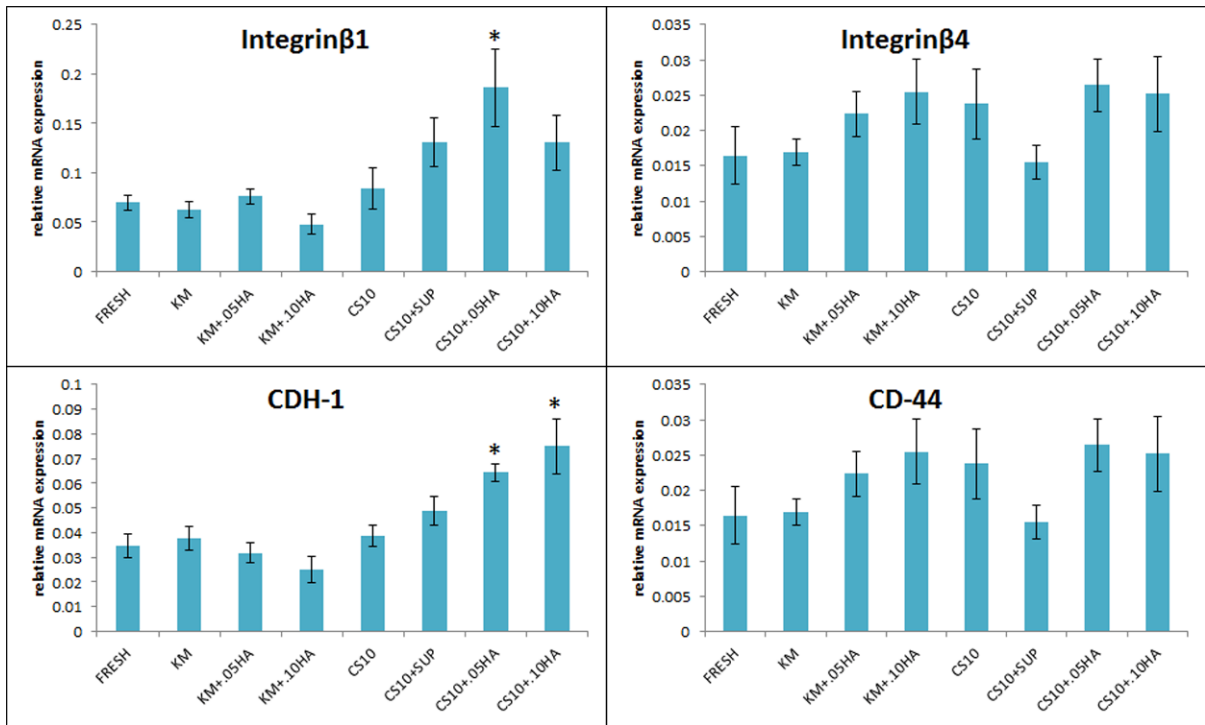


Figure 10: Relative mRNA expression normalized to GAPDH expression. Mean \pm standard error of the mean. Significance $*p < 0.05$ to Fresh samples. KM= Kubotas Medium with 10% DMSO and 10% FBS. CS10=cryostor, CS10+sup=cryostor10 with KM supplements. 0.05% and 0.10% refer to the HA% supplemented in each sample.

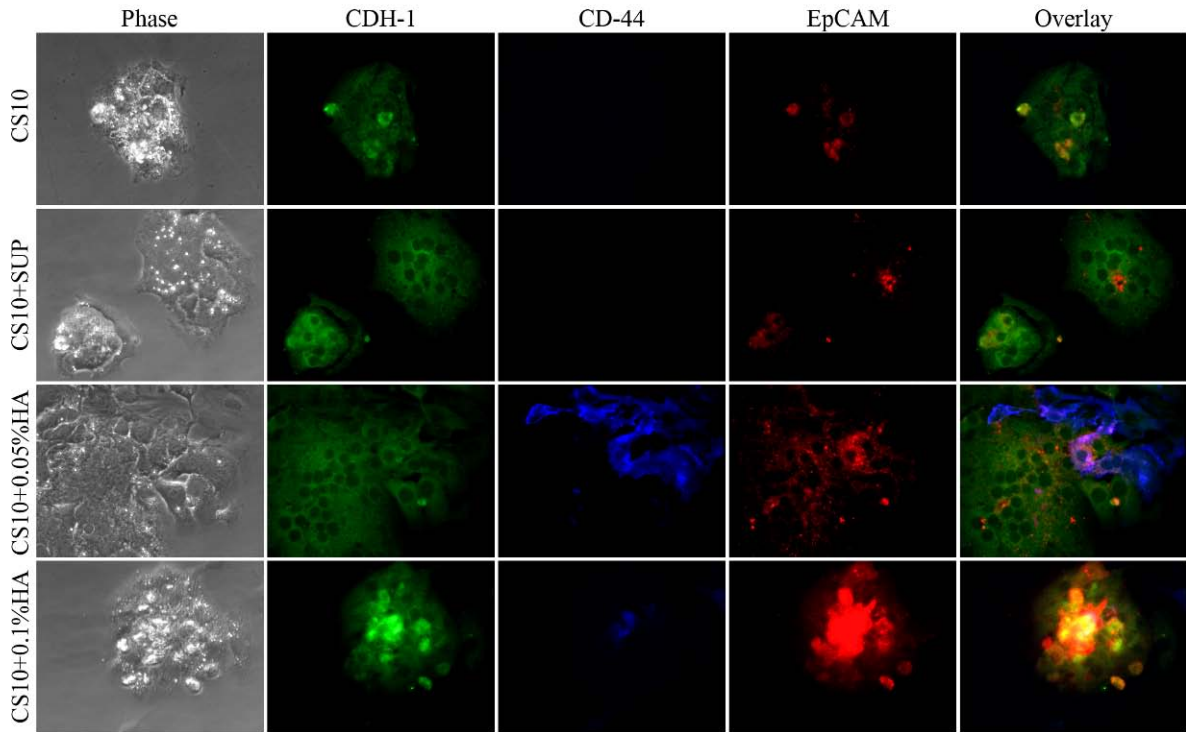


Figure 11: Cryopreserved hepatic progenitor cells after 24hr attachment on collagen III. CS10=cryostor, CS10+sup=Cryostor10 with KM supplements. 0.05% and 0.10% refer to the HA% supplemented in each sample.

Discussion

As previously described, the rate at which samples freeze is a key component in cryopreservation success. The freezing rate, in combination with components in the freezing media, affects the formation of ice and response to injury. A slow cooling rate leads to external ice crystals, causing mechanical lesions on the cell membrane, dehydrating the cells as water exits and solute enters. A fast cooling rate leaves small intracellular crystals in the cells, but does not dehydrate the cells and prevents larger external ice crystals. As water freezes intra- and extra-cellularly, solute and salts in the freezing media diffuse and are pumped across the membrane to find an osmotic balance. Cryopreservation depends on cell properties, such as membrane diffusivity, membrane permeability, surface area to volume

ratio as well as freezing membrane properties, such as internal and external osmolality, latent heat of fusion of ice, internal and external vapor pressure, leading different cell types to respond to cryopreservation differently and require different freezing methods. The cryopreservation of human hepatocytes show greatest success with a progressive freezing, with its slow freezing rate, following a 30 minute pre-incubation at 37°C and could potentially be used in cell banking [167]. Work has been done also involving the cryopreservation hepatic stellate cells [182], which are an important player in the successful culturing of human hepatic stem cells (hHpSC). Here, both cooling rates and cryoprotectants were investigated and have a clear effect on the resulting viability and cell survival. It was found that DMSO as a cryoprotectant gave superior attachment and viability for mature hepatocytes. More extensive studies of freezing mature rat hepatocytes in a controlled rate freezer with programmed steps found better control of viability using these controlled freezing steps [193]. The freezing program designed for this study took into account these freezing steps to minimize the latent heat seen in freezing, resulting in a smooth sample freezing rate and better viability.

The second key component to successful cryopreservation widely studied is the freezing media. Many studies standardly use the appropriate culture medium and supplement with cryoprotectants [181]. Bovine or calf serum and DMSO are commonly used as cryoprotectants, but both can initiate differentiation of early progenitor cells [175]. Serum cannot be used for samples intended for clinical applications, due to possible xenozoonosis contamination [194]. Univ of Wisc/Viaspan (UW) is designed for hypothermic tissue and organ storage, around +4°C, and contains high potassium, low sodium, as well as lactobionate, trisaccharide raffinose and mannitol, which together control swelling, osmotic

pressure and scavenge for radicals, as well as glutathione, adenosine, and allopurinol to facilitate ATP production [168]. CryoStor, similar to UW/ViaSpan, is isotonicly buffered for low temperatures (with low sodium and higher potassium), as opposed to culture temperature at 37°C [195, 196]. In cultures frozen in CryoStor freezing media, improved viability survival with less DMSO cryoprotectant than freezing with standard culture media was seen [172]. Similarly, hematopoietic stem cells from cord blood had more colony formation units after freezing in Cryostor versus freezing with dextran40, both with 10% DMSO [170]. Human hepatocytes frozen with in CryoStor when engrafted in immunodeficient NOD-SCID mice were able to retain function and reconstitute hepatic and biliary lineages [171].

Traditional culture mediums mimic interstitial fluid around cells and are designed for culturing at 37°C, whereas transport buffers like UW or cryo-specific media like CryoStor are designed for lower temperatures. Thus, in this study we looked at freezing with our traditional culture medium (KM) supplemented with 10%FBS and10% DMSO, compared to cryo-specific CS10. As a third medium condition, we looked at CS10 medium supplemented with the factors added to RPMI in our traditional KM medium (CS10+SUP), as explained in detail in the methods. As a whole, samples in cryospecific medium fared better than those in culture medium. Additionally, CS10 samples did not include FBS, allowing for its application in clinical programs.

As the freezing rate allowed for viability under all sample conditions tested, we focused on cell attachment and colony formation of the cells under the different conditions. The colony formation was best in cells frozen in KM or CS10 + 0.05% HA. We assume that

the surviving cells in KM were able to expand quickly with maintenance of this condition, but that more cells survived in CS10 + 0.05% HA.

Considerable importance has been found for the several adhesion molecules known to be expressed by the early progenitors and needed for attachment and colony formation after thawing [183]. These adhesion molecules were shown to be important in the cryopreservation, while others showed that apoptosis can occur in cells detached from their matrix [197]. Therefore, it was of interest that some of the conditions tested proved especially effective at preserving these adhesion molecules during freezing. Cells frozen in this study were freshly isolated from human fetal liver resulting in cell suspensions comprised of ~90% hHBS and ~1-2% hHpSCs. The integrins associated with the hHpSCs are $\alpha 6\beta 4$, whereas those associated with hHBs, are $\alpha 1$, $\alpha 5$, $\alpha 6$, $\alpha 9$, and $\beta 1$ but no $\beta 4$ [18, 198].

We found that there was indeed a significant increase in the cell-cell adhesion CDH1 when cultured with HA. Additionally, a co-expression of CD44 was only seen in attached colonies from samples frozen with a hyaluronan presence in the freezing medium. The integrin $\beta 4$ expression was similar in multiple freezing conditions suggesting that the hHpSCs are likely preserved. By contrast, there was a significant difference in the expression of integrin $\beta 1$ among the conditions implicating a greater sensitivity by the hHBs for cryopreservation; the best conditions for preserving this population was CS10 + 0.05% HA.

Conclusions

In summary, cryopreservation of freshly isolated human hepatic progenitor cells in isotonic media, such as the CS10 cryopreservation medium, proved effective and was further enhanced by supplementation with small amounts of HAs (0.05 or 0.10%). The HA supplementation improved cell attachment and colony formation due at least in part to improved expression of key cell adhesion factors in the stem cells and progenitor cell populations.

Further studies investigating the combination of media supplements known to be essential in the culturing of hepatic stem cells, in combination with hyaluronan supplements may increase viability and attachment. This study provides successful methods in primary hepatic progenitor cell cryopreservation, and should be implemented for more efficient banking methods in both research and potential therapy applications.

CHAPTER V

Maintenance of Human Hepatic Stem Cell Phenotype in Three Dimensional Hyaluronan Cultures

Rachael Turner, Eliane Wauthier, William Turner, and Lola M. Reid

Abstract

A strategy important to the future of liver regeneration therapies is the culturing in three-dimensional (3-D) environment, and the cells and matrix materials found within the stem cell niche are ideal combinations for tissue engineering applications. An ideal culturing method involves the use of human hepatic stem cells (hHpSCs) with expansion and differentiation potential to accommodate the numbers of cells required for these therapies. Hyaluronans (HA), naturally occurring glycosaminoglycans with non-immunogenic effects, are a logical scaffold for 3D cultures for both grafts and bioartificial organs. In this study, hepatic stem cells (hHpCs), were cultured and embedded in hyaluronan matrix combinations. Cell aggregates formed within the HA hydrogels, remained viable, and demonstrated a stable phenotype for hepatic stem cells after 3 weeks of culturing. The phenotype consisted of stable co-expression of EpCAM, NCAM, and albumin, and showed hepatic function through albumin and transferrin production and urea synthesis. Hyaluronans, combined with collagen III and laminin, maintain hepatic function and stem cell characteristics, proving to be a suitable three-dimensional scaffoldings for hHpSCs.

Introduction

Living tissue undergoes constant remodeling, and dissociated cells tend to reform their native structures under appropriate environmental conditions. Normal epithelial cells within solid tissues are anchorage dependent and require an extracellular matrix under three dimensional (3-D) structure conditions to guide normal physiological processes and regeneration [140]. *In vitro*, normal epithelial cells do not survive for long and do not function properly unless co-cultured with the appropriate mesenchymal cell partners [194, 195]. To escape the need for co-cultures, one must utilize the paracrine signals from that epithelial-mesenchymal relationship; the signals are comprised of specific extracellular matrix components and soluble signals. If the signals are not defined, then one can use “conditioned medium” produced by interactions between the particular epithelial and mesenchymal cells to yield a partial effect. [184, 196, 197].

Extracellular matrix is known to regulate the cell’s morphology, growth and cellular gene expression. Achieving similar tissue-specific chemistries may be achieved *ex vivo* by using purified extracellular matrix components. Many of these are available commercially, and when used under conditions in which cell-shape changes and porosity are enabled, are conducive to cell behavior mimicking that *in vivo* [34].

More specifically, hepatic development is induced in a step-wise, lineage-dependent process involving coordinate maturation of the epithelial and mesenchymal cells. The maturational process is accompanied by lineage-dependent paracrine signals [27]. Thus, there is now recognition that the epithelial-mesenchymal relationship is lineage dependent. In the case of liver, hepatic stem cells (HpSCs), the first lineage stage within the liver acini, are partnered with angioblasts [17]. These give rise stepwise to descendents of the two, with the

fates of the cells defined by the paracrine signaling that changes throughout differentiation of the partners. The HpSCs mature into hepatoblasts (HBs) partnered with hepatic stellate cell and endothelial cell precursors and then either into hepatocytes partnered with endothelia or into biliary epithelia partnered with stellate cells and then stroma. By defining the paracrine signaling between the partners at each stage, one can define conditions that will sustain the cells at that lineage stage and yield cells that behave functionally at an appropriate stage.

Human hepatic stem cells (hHpSCs) are multipotent cells located within the ductal plates of fetal and neonatal livers, or the canals of Hering in pediatric and adult livers. These cells range from 7-9 μm in diameter, have high nucleus to cytoplasmic ratio, and are extremely tolerant of ischemia as indicated by the fact that they can be found in livers of cadavers up to 167 hours (~7 days) after systolic death [22]. They form colonies with a morphology similar to that of embryonic stem (ES) cells in that they form tight clusters of cells, each one enveloped by angioblasts, and with hepatic stellate cell precursors located adjacent to the perimeter of the colony. The cells are capable of differentiation to mature hepatocytic and biliary epithelial cells both in culture and *in vivo* [17, 198]. They constitute ~0.5-2% of the parenchyma of livers of all age donors. The hHpSCs are SOX 17+, HES1+, cytokeratins 8/18/19+, CD133/1++ (prominin), Indian and Sonic Hedgehog+++, E-cadherin++, claudin 3+, N-CAM+++, albumin +/- and express telomerase mRNA as well as have telomerase protein in the nuclei [12, 17, 18, 27, 38]. These cells show low or no type I MHC antigens, are completely negative for all forms of P450s, ICAM-1, α -fetoprotein (AFP) for mesenchymal cell markers (CD146, desmin, α -smooth muscle actin), endothelial cell markers (VEGFr, Von Willebrand Factor, CD31), and hemopoietic cell markers (CD14, CD34, CD38, CD45, CD90, and glycophorin A).

The hepatoblasts (hHBs) are the immediate descendents of the hHpSCs, are the liver's transit amplifying cells, and located adjacent to the niche. They are larger (10-12 μm) with higher amounts of cytoplasm, are found *in vivo* throughout the parenchyma in fetal and neonatal livers, and are tethered to the ends of the canals of Hering in pediatric and adult livers [196, 199]. With age, these cells decline in numbers to <0.01% of the parenchymal cells in postnatal livers but are also shown to expand during regenerative processes. The hHBs have an antigenic profile overlapping with but also distinct from that of the hHpSCs. They are CK8/18/19⁺⁺, Ecadherin⁺⁺, CD133⁺, have sonic and Indian Hedgehog expression, have telomerase mRNA and protein within the nucleus (and higher telomerase activity levels than hHpSCs), and are negative for claudin 3, NCAM, and all the mesenchymal, endothelial and hemopoietic cell markers. They differ from hHpSCs by having higher expression of albumin⁺⁺ and de novo synthesis of MHC antigens, α -fetoprotein⁺⁺ (AFP), and fetal forms of P450s (e.g. P450A7).

The matrix chemistry associated with the parenchymal cells within the sinusoids is present in the Space of Disse, the area located between the parenchyma and the endothelia or hepatic stellate cells and their descendents, myofibroblasts. In addition to a change in cell maturity within the different zones of the liver, a parallel change in matrix chemistries is also observed. The matrix chemistry periportally in zone 1 is similar to that found in fetal livers and consists of type III and type IV collagens, hyaluronans, laminin, and forms of chondroitin sulfate proteoglycans with low sulfation. It transitions to a different matrix chemistry in the pericentral zone 3, containing types I, III, IV and XVIII collagens, plasma fibronectin, and unique forms of heparin proteoglycans [200-203].

More specifically, the matrix chemistry in the liver's stem cell niches, the canals of Hering, has been characterized partially and found to comprise hyaluronans, laminin forms that bind to $\alpha6 \beta4$ integrin (presumably laminin 5), type III collagen and unique forms of minimally sulfated chondroitin sulfate proteoglycans (CS-PGs). In the region adjacent to the niche and occupied by hHBs, one finds type IV collagen, forms of laminin binding to $\alpha\beta1$ integrins, sulfated forms of CS-PGs and low levels of heparan sulfate proteoglycans (HS-PGs). Type I collagen and more sulfated forms of proteoglycans occur with the appearance of the committed progenitors and later lineage stages of parenchymal cells.

The microenvironment of the stem cell niche minimizes the availability of matrix-bound growth factors and cytokines. As cells emerge from the niche, the adjacent matrix chemistry associated with the hHBs, the liver's transit amplifying cells, allows for the continuous presence of growth factors stabilized by binding to the proteoglycans. It is assumed that the proteoglycan/growth factor complexes are critical in dictating lineage restriction of the cells toward adult fates and in promoting the differentiation of cells.

Attention is focused on hyaluronan acid, or hyaluronans (HA), a key component in the extracellular matrix chemistry within the liver stem cell niche, found in extracellular matrix, on the cell surface and inside the cell [204]. HA is part of the glycosaminoglycan (GAG) family, and is composed of a disaccharide unit linked with a Beta 1-4, Beta 1-3 bonds between glucosamine and glucuronic acid structures. HAs are present in greater quantities at times of cellular expansion/proliferation such as embryogenesis, wound repair, and organ regeneration [205-210]. As a scaffolding component, HA has been linked to neural crest cell migration, cardiac development and prostate duct formation. Rapidly dividing epithelial cells show an increased binding activity to HA, and it is possible that transformation from

epithelial to mesenchymal morphologies take place via interactions mediated by HA binding. HA is most abundant in soft connective tissue, but is present in all tissues and body fluids [211, 212]. It has a natural water carrying capacity. It is anionic and bears a negative charge, attracting the positively charged hydrogen of water molecules. It is also thought to be conducive to migration [213]. The bifunctionality of the HA within the body suggests that removal and synthesis can be equally important in both morphogenesis and tissue homeostasis. The highest levels of HA are found within mesenchymal tissues, despite the fact that most cells are capable of synthesizing HA. Some have shown that HA is prominent in early liver injury models. Hepatic stellate cells produce HA in primary cultures and in liver injury. Their production of HA increases in parallel with expression of CD44. In addition, there is evidence of inhibitory effects of HA including the inhibition of cellular differentiation, and levels of HA in the blood have been correlated to hepatic regeneration [214].

In order to determine the mechanism by which HA influences tissue growth within the extracellular matrix, it becomes important to study the method of attachment of cells. Two forms of hyaluronan receptors have been found; CD44 found on the hepatic hHpSCs and LYVE-1 found on the angioblasts [215-217]. In addition, many integrins have been found in the ductal plate and associated with the hepatic hHpSCs, including $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 4$, but no $\alpha 1$ or $\beta 1$. Integrins associated with hHBs are $\alpha 1$, $\alpha 5$, $\alpha 6$, $\alpha 9$, and $\beta 1$ but no $\beta 4$. With the maturation towards hepatocytes there is a disappearance of integrin receptors that bind to laminin. In maturation towards biliary, there is an association with a maintenance of laminin-binding integrin receptors [218]. HA binds proteins in the ECM, on the cell surface and in the cytosol. Two cell-surface hyaluronan receptors, CD44 and

RHAMM, have a direct role for hyaluronans in regulating cell motility, invasion and proliferation. Most importantly, CD44, a cell surface protein, mediates cellular attachment of the liver cell progenitors to HA components of the ECM.

Currently, HAs are already being used clinically and are readily available and FDA approved. Hyaluronic acid is conserved across all species and is biocompatible, eliciting no inflammatory, immunologic or toxic response [219] . Immuno-neutrality and high water saturation combined with its ability to be easily modified, make it a great building block for biomaterials and use in tissue engineering therapies.

Essential in developing alternative therapies is understanding and utilizing the relationships between the epithelium and mesenchyme, as well as the signaling molecules and cellular matrix by making use of subpopulations of human liver cells with extraordinary expansion potential: human hepatic hHpSCs and their immediate descendents, hHBs, and committed progenitors. Extensive research has been done also on identifying the matrix materials found within the stem cell niche. The combination of the cells and matrix materials found within the stem cell niche makes them ideal for tissue engineering applications. In this study, we show that human hepatic hHpSCs can be cultured and expanded in hyaluronan hydrogels, with the combination of appropriate matrix components and companion cells found in the liver stem cell niche. By culturing the HpSCs embedded in matrix materials native to the stem cell niches, cells are exposed to sufficient signals needed for expansion and differentiation of liver tissue, forming tissue-like materials that may be further used in tissue engineering therapies. Results show that hHpSCs cultured in hyaluronan hydrogels combined with collagen III and laminin maintain hepatic function and further express stem cell characteristics.

Materials and Methods

Liver Sourcing and Processing.

Fetal liver tissues were provided by an accredited agency (Advanced Biological Resources, San Francisco, CA) from fetuses between 16-20 weeks gestational age obtained by elective pregnancy terminations. The research protocol was reviewed and approved by the Institutional Review Board for Human Research Studies at the University of North Carolina at Chapel Hill. Suspensions of fetal human liver cells were prepared as described previously [18, 225]. All processing and cell enrichment procedures were conducted in a cell wash buffer composed of a basal medium (RPMI 1640) supplemented with 0.1% BSA (BSA Fraction V; Sigma-Aldrich), insulin and iron-saturated transferrin (both at 5 ug/ml; Sigma-Aldrich), trace elements (300 pM selenious acid and 50 pM ZnSO₄), and antibiotics (AAS; Invitrogen). Liver tissue was subdivided into 3-ml fragments (total volume ranged from 2 – 12 ml) for digestion in 25 ml of cell wash buffer containing type IV collagenase and deoxyribonuclease (both at 6 mg per ml; Sigma- Aldrich) at 32°C with frequent agitation for 15 – 20 min. This resulted in a homogeneous suspension of cell aggregates that were passed through a 40-gauge mesh and spun at 1,200 RPM for 5 min before resuspension in cell wash solution. Erythrocytes were eliminated by either slow-speed centrifugation or by treating suspensions with anti-human red blood cell antibodies (1:5,000 dilution; Rockland) for 15 min, followed by LowTox Guinea Pig complement (1:3,000 dilution; Cedarlane Labs) for 10 min, both at 37°C. Estimated cell viability by Trypan blue exclusion was routinely >95% [33, 189, 226].

Hepatic Stem Cell Culture Conditions.

For all cultures, Kubota's medium, specially designed for the expansion of hepatic progenitors, was used. KM was developed originally for rodent hepatoblasts [227] and subsequently found effective for hHpSCs and hHBs [25, 37, 189, 226]. KM consists of any basal medium, here being RPMI 1640 (Gibco /Invitrogen, Carlsbad, CA) with no copper, low calcium below 0.5 mM (here being 0.3 mM), trace elements (zinc--10-12M and selenium—10-9M), insulin (5 µg/ml), transferrin/fe (5 µg/ml), high density lipoprotein (10 µg/ml) and a mixture of free fatty acids bound to bovine serum albumin. Detailed methods for its preparation are given in a methods review [33].

The suspensions of fetal human liver cell mix, highly enriched for hepatoblasts, were suspended into Kubota's Medium, and the cell mix was plated at ~300,000 cells per 100mm² round culture dish (78.5cm² surface area). The low cell concentration allows plenty of empty room around stem cells for colony growth, and reduces/slows down the chance for non-parenchymal cells from forming fast growing confluent groups that can take over the cultures. The low cell concentration also prevents mature hepatocytes from forming confluent groups and having the necessary signals to remain in culture. This is complemented by the media conditions that are non-permissive for mature hepatocytes. Cells are plated with 10% fetal bovine serum (FBS) for 20-24 hrs to facilitate attachment. Afterwards cell media is changed every 4-6 days with serum-free HK media.

Typical plates have single cells and small clusters of cell that adhere after the initial 24hrs. Colonies begin to appear after 1-2 weeks. Non-parenchymal cells, found to be a mix of hepatic stellate cell and endothelial cell precursors, also continue to grow on the plate,

sometimes around the borders of colonies as they grow forming a smooth supporting edge, or sometime forming large confluent tight areas that colonies grow on top of.

HA matrix Preparation.

All hyaluronan materials are commercially available from Glycosan Biosciences (Salt Lake City, Utah --now Biotime, Alameda, CA), and consist of thiol-modified carboxymethyl HA (or CMHA-S), a chemically modified HA derivative with disulfide bridges for cross-linking. The cross-linking is initiated by a PEGDA crosslinker, enabling the hydrogels to be formed in the desired container [219, 223-226]. The level of crosslinking activity and stiffness of the hydrogel can be regulated by the amount of PEGDA added. The hydrogel substrata were constructed by dissolving dry reagents in KM to give a 2.0% solution (weight/volume) for the HA gels and the PEGDA crosslinker is dissolved in KM to give a 4.0% weight/volume solution. Samples were then allowed to incubate in a 37° C water bath and allowed to completely dissolve. Collagen III and laminin samples are available from Sigma (St. Louis, MO) and were used at a concentration of 1.0mg/ml. A ratio of 1:4 is applied to blend the crosslinker and hydrogels. At the end of the experiment, hydrogels were dissolved easily using dithiothreitol reducing agents with hyaluronidase without damaging the cellular component.

Cell matrix culture conditions.

Cells were isolated from cultured human fetal liver samples as described in the general methods. After three weeks in culture, stem cell colonies, approximating 3-5,000

cells/colony, were picked and put into suspension. Cell suspensions of 200,000 cells were then combined with matrix mix. PEGDA crosslinker added, and the cell matrix material immediately added to wells in a 4 well chamber slide. Once the gel sets, an equal amount of media, spiked with 0.03mM NH₄, was added to the top of the well. Cultures were then maintained for a period of 21 days, with media changes every 48hrs. The culture media was saved for analyses. Multiple runs were performed with different liver samples to ensure consistency.

Viability Assay

Viability was assessed in cultures using Molecular Probes Calcein AM live cell viability kit (Molecular Probes, Eugene Oregon). Membrane-permeant calcein AM was cleaved by esterases in live cells to yield cytoplasmic green fluorescence.

Histology and Sectioning

Samples of hHpSC-seeded HA hydrogels were fixed overnight with 4% buffered paraformaldehyde. Fixed samples were embedded in HistoGel™ specimen medium, with the resulting construct transferred into a cryomold and embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) for flash freezing. Samples were submitted to the Histology Research Core Facility at UNC Chapel Hill for serial cryosectioning at 15 μm section thickness. Thinner sections were not feasible due to the nature of the hydrogel handling properties. Frozen sections were stored at -80°C until staining, at which time they

were stabilized to room temperature before PBS washing at the beginning of immunochemistry protocols.

Immunohistochemistry

Sections were first washed with PBS, bordered using a PAP-PEN, and blocked for using PBS + 0.1% Triton + 10% goat serum. Primary antibodies were applied and allowed to incubate, followed by another wash and the application of secondary (Alexa Fluor) antibodies. Once tagged, a mounting media containing DAPI was applied and slides are imaged using confocal fluorescent microscopy. For a list of primary and secondary controls, please see Table 6. Positive control cell lines are also listed in **Table 6**, and samples stained with only secondary antibodies were used as negative controls.

Table 6: Antibodies and respective positive control cell lines used for immunohistochemistry.

Marker	Description	Manufacturer (Cat. No.)	Source (Isotype/Emission)	Stock Concentration	Titer	Positive (Cell Line)
EPCAM (human)	Epithelial cell adhesion molecule	Lab Vision/Neomarkers (MS-181-P1)	mouse anti-human (IgG ₁)	200 µg/ml	1:500	Hep3B
NCAM (human)	Neural cell adhesion molecule	BD Pharmingen™ (559043)	mouse anti-human (IgG _{2B})	1 mg/ml	1:500	SK-N-SH
IgG _{2B} (mouse)	Alexa Fluor® 488	Invitrogen™ Molecular Probes® (A21141)	goat anti-mouse (488 nm)	2 mg/ml	1:800	N/A
IgG ₁ (mouse)	Alexa Fluor® 568	Invitrogen™ Molecular Probes® (A21124)	goat anti-mouse (568 nm)			

Confocal Microscopy

Histological sections of hHpSC-seeded HA hydrogels stained and tagged with secondary antibody fluoroprobes were identified using laser confocal microscopy. Negative

controls were used to normalize backgrounds and eliminate image acquisition noise. Microscopy was performed at the Michael Hooker Microscopy Facility and UNC-CH. Equipment consisted of a Leica SP2 Laser Scanning Confocal Microscope equipped with a Hg/Xe arc lamp for epi-illumination, excitation lasers at wavelengths of 350/364 nm (UV), 488/476/488/514nm (Blue Ar laser), 561 nm (Green Solid State diode pump laser) and 633 nm (Red HeNe laser, visible red), acousto-optical beam splitters (AOBS) and tunable filters (AOTF), photo-multipliers tube (PMT) light detectors with spectral discrimination and a high-precision galvanometer z-axis positioning stage. LCS Software was used for image acquisition and processing.

Analysis of secreted protein production

Concentration levels of secreted albumin, transferrin, and urea in culture media were measured to determine hepatic functions of hHpSC in the different hydrogel formulations during 1 week of culture. Media supernatant was collected daily after 48-hour incubation starting on day 2 post-seeding and stored frozen at -20°C until analyzed. Albumin production was measured by ELISA using human albumin ELISA quantitation sets with respect to human reference serum standards from the quantitation set manufacturer (Bethyl Laboratories, Montgomery, TX) in terms of horseradish peroxidase (HRP) -conjugated fluoroprobe levels (detection antibody against albumin) by colorimetric absorbance at 450 nm. Urea production was analyzed using blood urea nitrogen colorimetric reagents with respect to reference standards from the reagent manufacturer (Bio-Quant Diagnostics, San Diego, CA) by colorimetric absorbance at 630 nm. All assays were measured individually

with a cytofluor Spectramax 250 multi-well plate reader (Molecular Devices, Sunnyvale, CA).

Quantitative Real Time PCR (qRT-PCR).

Gene-specific primer sequences for quantitative gene expression analysis of differentiation markers [17, 33, 189, 190] were designed and validated for PCR doubling efficiency and priming specificity using cDNA templates assembled from Total RNA of relevant cell lines (listed in **Table 6**) positively expressing each gene of interest and extracted with the RNeasy Mini Plus kit (QIAGEN). Quantitative Real Time PCR (qRT-PCR) measurements were performed with an Applied Biosystems® 7500 Real-Time PCR System available in the Functional Genomics Core Facility at UNC Chapel Hill. Primers used can be found in **Table 7** All measurements of relative expression were normalized with respect to GAPDH by the absolute quantification method described elsewhere [191, 192].

Table 7: Primer sequences used for quantitative RT-PCR.

Gene	Description	NCBI Ref. Seq.	Primer Sequence (5' → 3')	
			Forward	Reverse
GAPDH	Glycerine aldehyde-3-phosphate dehydrogenase	NM_002046.3	AAGGTGAAGGTCGGAGTCA A	AATGAAGGGGTCATTGATGG
AFP	α -fetoprotein	NM_001134.1	CCATGAAGTGGGTGGAATCA A	TCTGCAGTACATTGGTAAGAATCCA
EPCAM	Epithelial cell adhesion molecule	NM_002354.1	GACTTTTGCCGCAGCTCAGG AAG	GCCAGCTTTGAGCAAATGACAGTATTT TG
NCAM	Neural cell adhesion molecule	NM_000615.5	GCGACCATCCACCTCAAAGT	CTCCGGAGGCTTCACAGGTA

Results

Human hepatic stem cells remain viable and maintain phenotype for weeks in HA hydrogels.

hHpSCs were sustained in HA hydrogel conditions for 3 weeks in static cultures with KM, replaced and collected every 48hrs. **Figure 12** shows cells within HA gels at 3 weeks. The cells are viable as indicated by staining with Calcein AM (green).

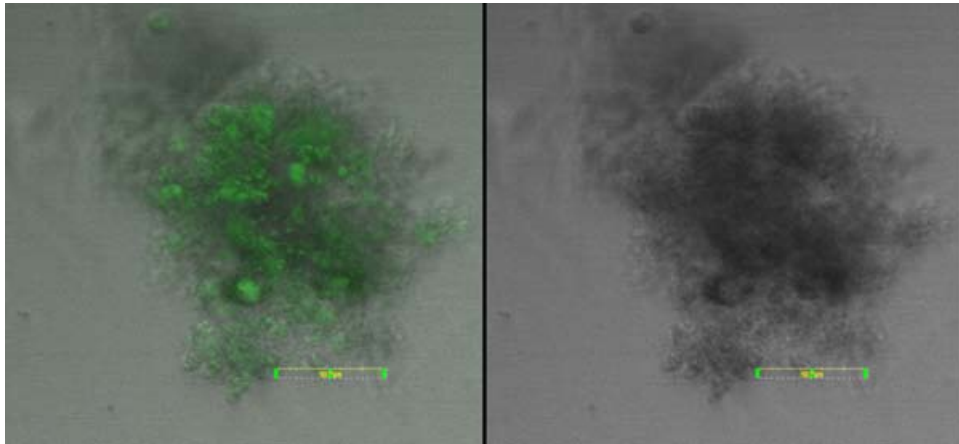


Figure 12: hHpSCs in HA hydrogels at 21 days in culture. Green Calcein AM fluorescence shows viable cells.

After 3 weeks of culture, cells were analyzed for genetic expression. Levels of mRNA expression (**Figure 13**) were normalized to GAPDH. All measurements are expressed as fold changes compared to initial hepatic stem cell colonies prior to three-dimensional culture in hyaluronan hydrogels. In both experimental hyaluronan culture conditions (HA and HA+collagen III+laminin), there is a significant increase in EpCAM (7.72 ± 1.42 , 9.04 ± 1.82) and Albumin (5.57 ± 0.73 , 4.84 ± 0.84) when compared to initial colony expression. There was also a significant decrease in the hepatoblast differentiating marker AFP in both conditions (0.55 ± 0.11 , 0.17 ± 0.03). In addition, the HA+CIII+Lam condition showed a significant decrease in AFP expression when compared to the basic HA culture.

In addition to genetic expression analysis, 3D cultures were fixed and prepared for immunohistochemical staining, and confocal microscopy imaging was used to determine how specific differentiation markers were distributed within the cultures. For both hyaluronan cultures, there was colocalization of EpCAM and NCAM (**Figure 14**). In hydrogel cultures supplemented with additional CIII and Laminin, the EpCAM signal was shown to be stronger than those with hyaluronans alone. This is consistent with the previous findings regarding mRNA expression levels in these 3-D culture conditions.

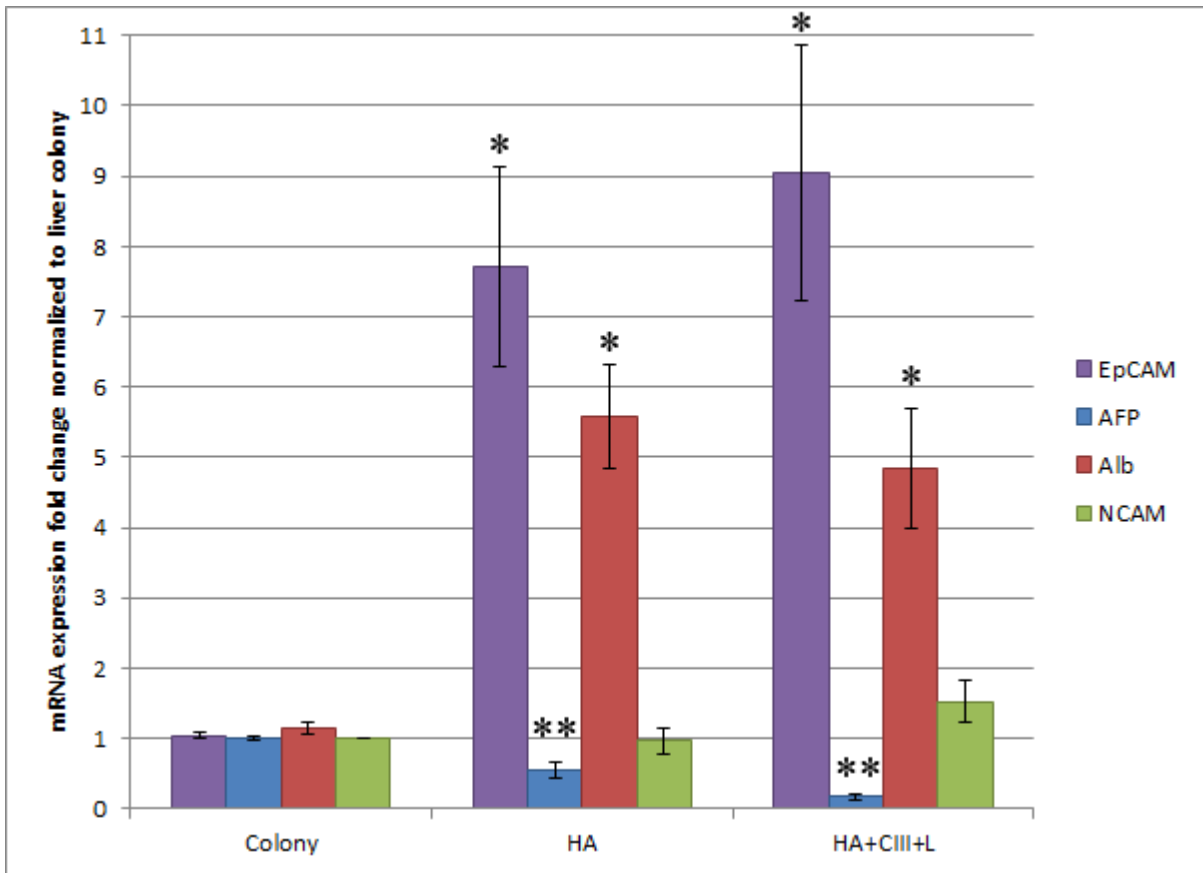


Figure 13: Gene expression of hepatic stem cell phenotype markers. Expression levels are normalized to GAPDH expression, and fold changes are normalized to initial expression in colonies. * denotes $p < 0.05\%$ significance between experimental condition and initial colony expression. ** denotes $p < 0.05\%$ significance between experimental condition and initial colony expression as well as significant expression between the two experimental conditions.

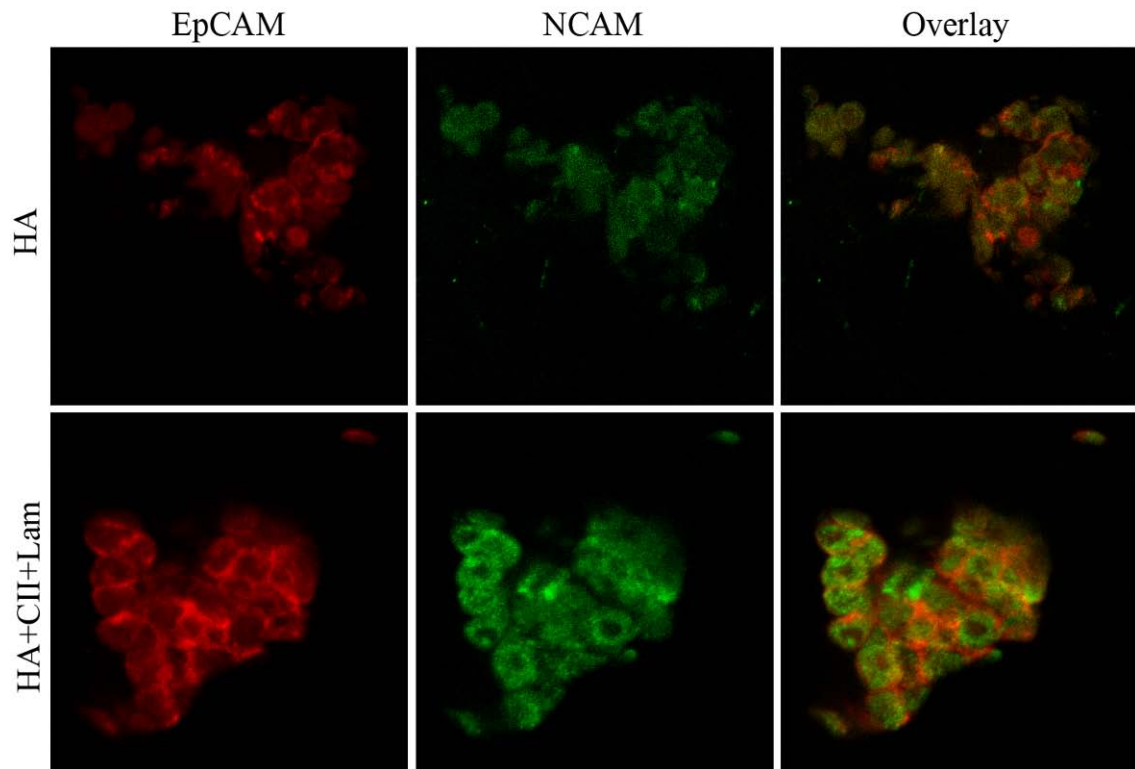


Figure 14: expression of EpCAM and NCAM in three-dimensional HA colony sections.

Functional Assays of Hepatic Metabolism for hHpSCs seeded in hydrogels.

Immunosorbent assays were conducted on media samples collected during culture to determine absolute concentrations of secretory proteins that define hepatic function (**Figure 15**). Albumin, Transferrin, and Urea concentrations were normalized per cell. Throughout cultures, hHpSCs in both hydrogel compositions secreted albumin and transferrin at increasing concentrations, while urea synthesis equilibrated to comparable levels in all hydrogel conditions.

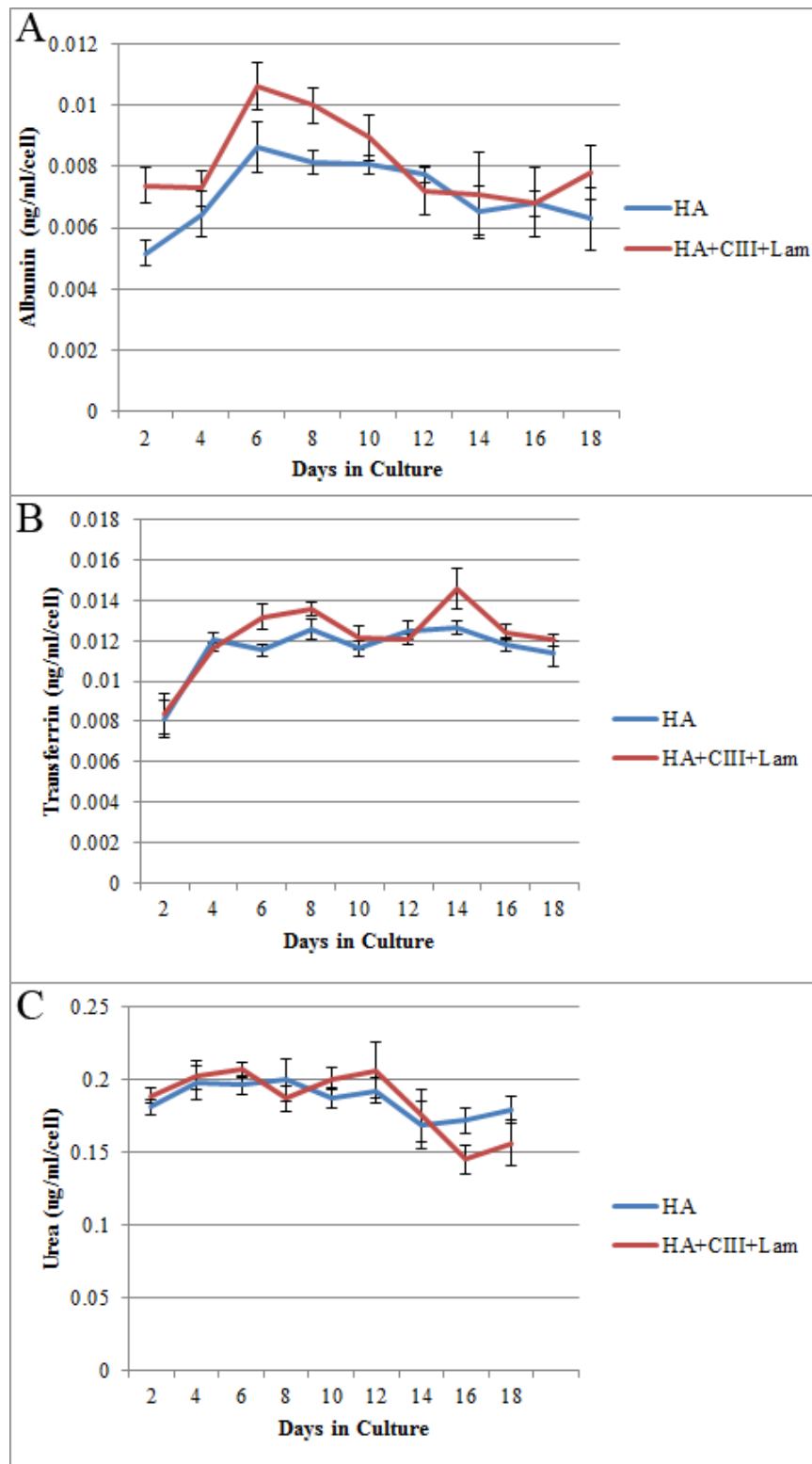


Figure 15: functional assays of hepatic function over time. A) albumin, B) Transferrin, and C) Urea in three-dimensional hyaluronan culture over time for levels are normalized per cell.

Discussion

Human hepatoblasts (hHBs) seeded into the HA hydrogels were found to retain their viability and their ability to divide for weeks, more than 3 times longer than those on plastic. Although other culture conditions are permissive for survival of the hHpSCs (e.g. culture plastic, angioblast feeders, type III collagen, uncrosslinked HAs), hyaluronans have been the only culture condition identified that facilitate survival, proliferation and maintenance of hHBs. The hHBs do survive on STO embryonic stromal feeder cells but go essentially into growth arrest and undergo differentiation towards more mature fates [232].

EpCAM is a common marker of hHpSCs and hHBs at both gene and protein expression levels. It is characteristically found throughout the hHpSCs but restricted to the plasma membrane in hHBs [33, 189, 226]. This data shows increased overall expression of EpCAM beyond established levels for hHpSC colonies on plastic plates. This may be attributed both to the matrix chemistry as well as the 3D structure condition.

In culture, AFP is not expressed in hHpSCs at all, but is characteristic of hHBs and gradually fades with maturation to committed progenitors and later lineage stages [17, 184, 185, 228]. The significant decrease in AFP expression in both hyaluronan conditions can be interpreted either that the cells remain as hHpSCs or that they mature to later lineage stages at which AFP is not expressed. The maintenance of NCAM, a marker of stem cells, but not mature cells, implicates that the former interpretation is correct. This suggests that the matrix chemistry has an influence in maintaining the hHpSC phenotype, as cultures supplemented with collagen III and laminin in addition to the 3D hyaluronan structure saw decreased AFP expression. The albumin expression seen in both hyaluronan conditions reflect an increased function of hHpSCs in a 3-D environment.

The rigidity of HyStem hydrogels can easily be adjusted, and depending on the application, the stiffness can have an impact on the phenotype, growth and differentiation of the stem cells or progenitors in cultures [75, 234, 235]. For these studies, the standard recommended formulation of HyStem hydrogels was used and kept constant throughout the study. Previously reported in Lozoya et al [235], the hepatic progenitor phenotype is conserved at a rigidity below 200Pa. The hyaluronan composition used in this study was recorded to be 25Pa, well below levels for induced mechanical differentiation.

These hyaluronans are also biocompatible and biodegradable [224, 236]. This was observed in the cultures. After approximately 1 week in culture, it was apparent that cells began breaking down the hyaluronans, and some material was lost in the media changes, including a loss of cells. This is apparent in the serum protein analyses, where a decrease in albumin and is shown after a week; as the total number of cells is decreased due to the loss of material during media change, so does the level of produced albumin. This biodegradation is extremely beneficial in future *in vivo* investigations in which hydrogels will provide an important initial structure and microenvironment for transplanted cells, but it is also expected that cells will eventually replace the scaffolding used for the transplantation with matrix components appropriate for integration into the host tissue to promote regeneration.

In response to the limitations observed with traditional liver transplants for liver failure, efforts by numerous investigators are ongoing to develop alternative liver cell-based therapies [237]. Co-culturing and injecting with a cell suspension allows for solidification *in situ* that can fill any cavity space. The use of hyaluronic acid as a material for injectable material for tissue engineering proves to be promising due to its long-lasting effect while maintaining biocompatibility [238, 239]. Cross-linking methods also maintain the material

biocompatibility, and its presence in extensive areas of regenerative or immature tissue areas make it a very attractive injectable material.

Hyaluronan hydrogels, in combination with a serum-free medium tailored for hepatic progenitors (KM medium), have proven suitable three-dimensional scaffoldings for human hepatic progenitors, particularly for hHpSCs if uncrosslinked, and for hepatoblasts, with cross-linking to specific rigidity conditions that are permissive. They maintain the cells as hHBs in terms of viability and phenotypic stability through prolonged culture periods, and with minimal lineage restriction towards either biliary or hepatocytic fates.

CHAPTER VI

Location determination of transplanted hepatic progenitors using Positron Emission Tomography (PET) and Luminescent Imaging

Rachael A Turner, Randall McClelland, Jim Bowsher, Elaine Wauthier, Lisa Samuleson, David Gerber, Edward Shu, and Lola M Reid

Abstract

Limitations with current cell transplant methods have indicated a need for more efficient grafting methods in the newly developed methods for regenerative medicine. In this study, we use two methods, radiolabeled positron emission tomography (PET) imaging and bioluminescent optical imaging, to look at cell localization post-transplant in murine models. Results show that better engraftment and localization is seen when cells are grafted to the target tissue using an injectible biomaterial in liver cell therapies.

Background

Acute liver failure caused by metabolic errors, viral hepatitis, and drug-induced and toxin-induced liver diseases are often lethal to patients if not treated quickly. Currently, the only available treatment for these patients is orthotopic liver transplantation. However, these transplants are expensive and extremely invasive. Together with the increasing number of impending transplants and the limited availability of donor livers, a need for alternative therapeutic strategies is presented.

Current research focus has been on the use of cell transplantation as an alternative therapy, or as a bridge towards transplantation. Cells are isolated from donor tissue not usable for whole organ transplants. The goal of regenerative medicine is to restore tissue function by transplanting exogenous cells. One of the hurdles in using the cells is establishing methods for transplanting the stem cells or progenitors back into the hosts. Results from these clinical trials have been encouraging, but the promise is compromised by the lack of growth observed of transplanted mature cells, their high immunogenicity (necessitating immuno-suppression), and the need to use freshly isolated cells [235]. Typical injections are by way of portal vein cannulation or into ectopic sites like the red pulp of the spleen. The cells distribute to many tissues, not just the target tissue, and often lead to emboli that translocate into secondary vascular beds such as those in the lungs [236-239].

The development of grafting technologies, where transplanted cells are delivered as an aggregate on or in scaffolds that can be localized to the diseased tissue, should be promising technologies that promote necessary proliferation and engraftment. The cells to be transplanted need to be delivered as an aggregate or on or in scaffolds that can be patched onto/into the tissue.

Availability of stem cell populations from solid human organs, such as liver, for use in clinical programs in cell therapies has made necessary the development of non-invasive cell labeling and tracking methods to monitor medical treatments. These therapies require stem cells to expand in order to replace diseased tissues in support of failing organs. The goals of the current study are to identify effective conditions for optimal hepatic stem cell expansion and localization, and to develop marking methods of the stem cells for detection and monitoring after *in vivo* transplantation. We have investigated both PET and optical luminescence imaging to localize cells during cell transplantation and cell grafting. Both techniques include the infection of donor cells with a virus expressing a specific imaging tag prior to transplantation.

Results and Discussion

PET studies were done by first developing a lentiviral vector expressing thymidine kinase, with a CB promoter. For transplantation *in vivo*, cells (1.5×10^6 labeled cells) were injected into the liver via the spleen. To provide an imaging signal, a radiolabeled probe [(18)fluoro-3-hydroxymethylbutyl) guanine ([18F]FHBG) was exploited to provide evidence of cells marked with Thymidine Kinase (TK). As FHBG enters the cell, it is cleaved by TK and trapped inside the cytoplasm – inducing PET signals of accumulated FHBG. The probe is injected into animals 3 hours prior to imaging, allowing for excess FHBG not cleaved by TK producing cells to circulate the body and collect in the kidneys and bladder. Results, seen in **Figure 16**, show that after 22 and 85 days, cell signal is not only present in the liver, but also present in the lung, spleen, and kidney tissue. Thus, the cells engrafted partially into the liver but dispersed also to multiple ectopic sites, and those cells at

ectopic sites did not die, as reported (or assumed) by many to occur, but survived for months within those ectopic tissues.

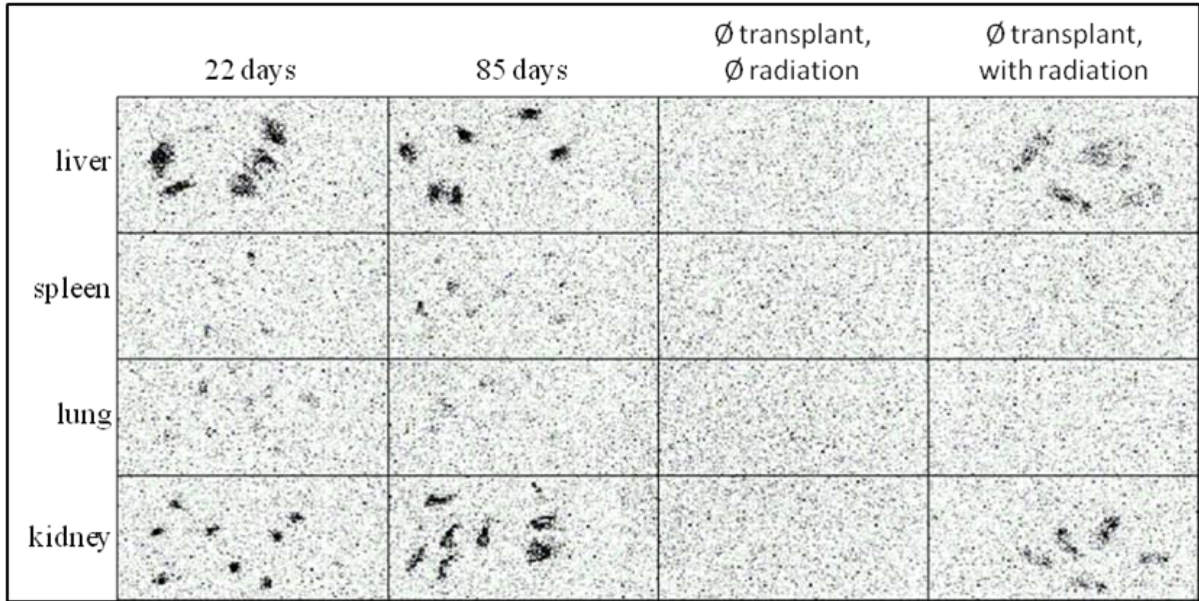


Figure 16: PET imaging of FHBG concentration activity in murine hosts injected via vascular route with human hepatic stem cells infected with TK. Positive signal is seen in liver, spleen, lungs, and kidney at 22 and 85 days post-injection.

An alternative imaging method was also employed using a bioluminescent marker. To test efficiency of cell localization using grafting strategies, cells were first infected with a bioluminescent marker. After cell isolation, hepatic progenitor cells were infected for 4 hrs at 37°C with a luciferase-expressing adenoviral vector at 50POI (Vector Biolabs, Philadelphia, PA). Mouse hepatic progenitor cells were isolated from a host C57/BL6 mouse. Mice were anesthetized and survival surgery was performed, opening the abdomen and slowly injecting 1.5x10⁶ cells directly into the liver lobe, via cell suspension or grafted using Hystem Hyaluronans (Glycosan) crosslinked with Poly (Ethylene Glycol)-Diacrylate (PEG-DA). Grafting materials selected for the graft should elicit the appropriate growth and

differentiation responses required for successful cell transplantation [245]. For imaging, mice were injected subcutaneously with luciferin, 10-30 minutes prior. Luciferin was oxidized in the presence of the cells infected with luciferase, producing oxyluciferin and energy in the form of light. Using an IVIS Kinetic optical imager, the bioluminescent signal was detected with the CCD camera and localized within the mice.

In animals where cells were injected without grafting, cells were found both in the liver and lung at 24 hrs. At 72hrs, cell signal greatly decreased, leaving only a weak signal in the liver. However, when cells were grafted using hyaluronans, cells were localized to the liver, and remained viable and continued to produce a luminescent signal even after 2 weeks (**Figure 17**).

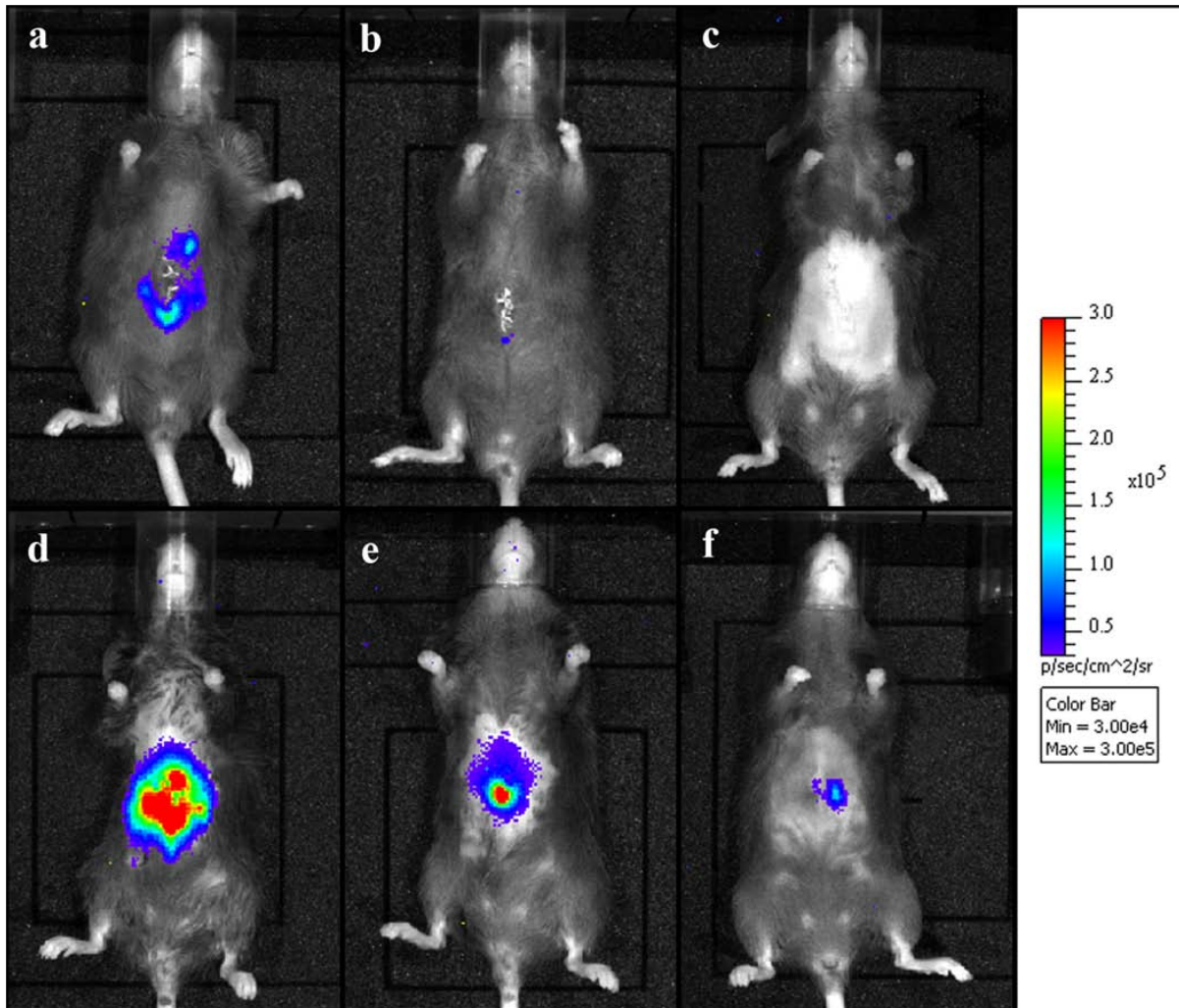


Figure 17: Transplantation of cells in murine livers . Injected cell suspension at a) 24 hrs and b) 72 hrs. c) control- no cells. Injectable cell graft at d) 24 hrs, e) 72 hrs, and f) 2 weeks post-transplantation.

Both methods used for imaging have their advantages in detecting and localizing cells in transplantation studies. PET imaging methods, using a radiolabeled probe, allows for extremely specific and detailed imaging of cells. Bioluminescent imaging does not allow for the same detail seen in PET imaging, but does allow for serial imaging in vivo.

As shown in both PET and Bioluminescent imaging studies, it was shown that cells injected without grafting methods were not localized only to the target liver tissue but were

present throughout the body. Moreover, the cells in ectopic sites survived for months. Not only does this lead to inefficient therapies, it raises the concern of what the cells at the ectopic sites might do and whether they might lead to pathologic conditions. Grafting with appropriate materials can optimize engraftment of transplanted cells in a tissue, prevent dispersal of the cells to ectopic sites, enhance the ability of the cells to integrate within the tissue as rapidly as possible, and should be applied to future tissue engineering and transplantation therapies.

Methods

Media and Solutions.

Kubota's Medium was designed originally for rodent hepatoblasts [246] and then found effective for human hepatic stem cells (hHpSCs) and human hepatoblasts (hHBs) [18], for human biliary tree stem cells (Wang et al, submitted), and for human fetal pancreatic progenitors (Y.F. Wang and L.M. Reid, manuscript in preparation). It consists of any basal medium (here being RPMI 1640) with no copper, low calcium (0.3 mM), 10^{-9} M Selenium, 0.1% serum albumin, 4.5 mM nicotinamide, 0.1 nM zinc sulfate heptahydrate (from Specpure, Johnson Matthew Chemicals, Royston, England), 10^{-8} M hydrocortisone, 5 μ g/ml transferrin/Fe, 5 μ g/ml insulin, 10 μ g/ml high density lipoprotein, and a mixture of free fatty acids that are added bound to purified human serum albumin. All media were sterile-filtered (0.22- μ m filter) and kept in the dark at 4°C before use.

Human fetal liver processing

Fetal liver tissues were provided by an accredited agency (Advanced Biological Resources, San Francisco, CA) from fetuses between 16-20 weeks gestational age obtained by elective pregnancy terminations. The research protocol was reviewed and approved by the Institutional Review Board for Human Research Studies at the University of North Carolina at Chapel Hill. Suspensions of fetal human liver cells were prepared as described previously [18, 225]. Briefly, processing was conducted in RPMI 1640 supplemented with 0.1% bovine serum albumin, 1nM selenium and antibiotics. Enzymatic processing buffer contained 300U/ml type IV collagenase and 0.3 mg/ml deoxyribonuclease at 32°C with frequent agitation for 15-20 min. Enriched suspensions were pressed through a 75 gauge mesh and spun at 1200 RPM for 5min before resuspension. Estimated cell viability by trypan blue exclusion was routinely higher than 95%.

Mouse liver processing

Liver cells were isolated using a modification of the 2-stage liver perfusion technique described by Seglen and employing the use of perfusion of calcium-free buffer containing EGTA to flush the liver and then Liberase TL (Roche) perfusion for 8 minutes to disassociate cells [247]. The murine hepatic progenitors, mHPCs, were separated from mature hepatocytes using gravity separation and centrifugation. Full details of the isolation and characterization of the mHPCs have been previously described [248]

Lentiviral construct and transfection

The infection with the lentiviral vector (**Figure 18**) was performed in cell suspensions after liver processing. Polybrene was added for 5 to 10 min before the infection. This positively charged molecule reacts with the negative charges of the sialic acid residues on glycans at the cellular membrane and diminishes the repulsive forces that block anchorage of viruses at the cell surface. Cells were placed in buffer (cell wash or hormonally defined medium, HDM) in an Eppendorf tube, spun for 5min at 1,200 rpm, and the supernatant was discarded. The cells were resuspended with HDM by gentle swirling. Polybrene was added at 2 ug/ml (final concentration) and the cells were allowed to sit at room temperature for 5 to 10 min. The virus was add (~100 ul/ 2 X 10⁶ cells) to the cells with polybrene and again allowed to sit first at room temperature for 1 hour and then on ice for 3 hours. At the end of the incubation, fresh medium was added and spun to eliminate free viral particles in the supernatant. The cells were plated at 100,000 cells/well of 6 well plates in HDM+10% fetal bovine serum (FBS). After 6-12 hours, the medium was changed to serum-free HDM and monitored for fluorescence. The ratios used were: NB1: 2x10⁶ cells/ 100ul HDM/polybrene (2ug/ml final)/100ul viral prep .

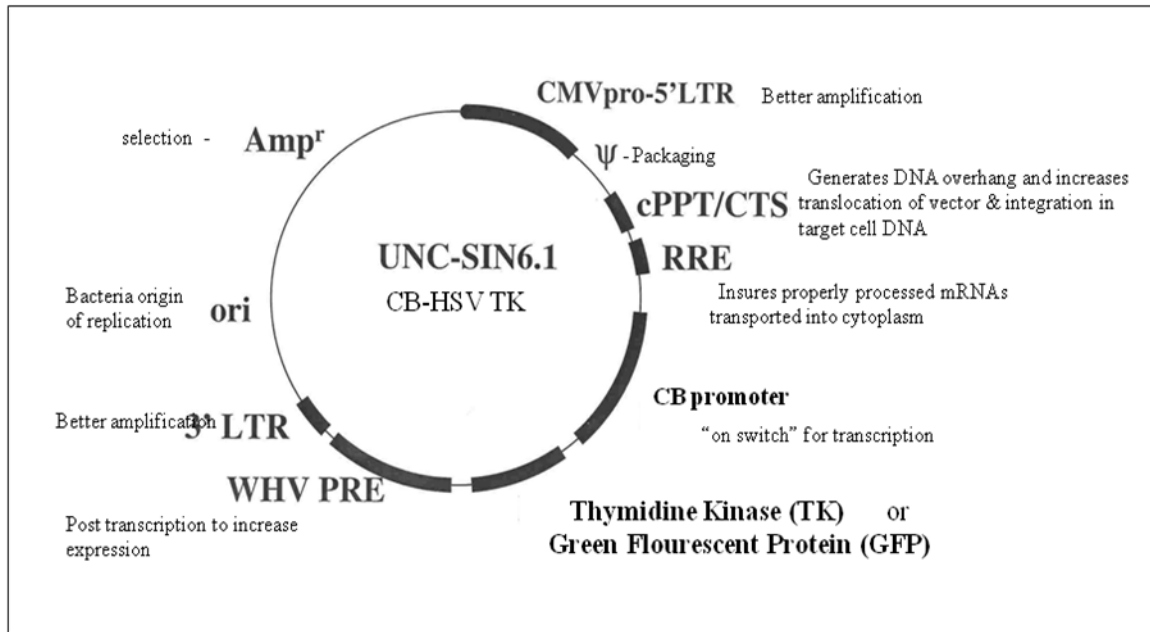


Figure 18: Lentiviral vector used to express thymidine kinase in implanted cells.

Mice.

C57 Bl 6 SCID/nod mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed in a barrier facility on the campus of the University of North Carolina-Chapel Hill. C57/black6 mice were bred in-house. Animals received care according to the Division of Laboratory Animal Medicine, UNC-CH guidelines, ones approved by AALAC.

In vivo Engraftment

Human hepatic stem cell engraftment: C57BL/6 SCID/nod mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used at ~5 weeks of age. Mice were anesthetized with Ketamine-HCl (Vedco Inc, St. Joseph, Mo) and Xylazine-HCl (ProLab LTD., St. Joseph, Mo) and injected intrasplenically with 8×10^5 cells. The spleen was

exteriorized through a small left flank incision (5 – 10 mm), and 70 µl of cell suspension was injected slowly into it using a 26-gauge needle on a Hamilton syringe. The spleen was returned to the abdominal cavity and the incision site was closed. **Murine Hepatic progenitor cell engraftment:** C57/bl6 mice (4-16 weeks) were anesthetized using ketamine (90-120mg/kg, Bioniche Pharma, Lake Forrest IL), and xylazine (10mg/kg, Akorn, Decatur, IL). Survival surgery was performed, opening the abdomen and slowly injecting material into the front liver lobe. The incision site was closed and animals were given 01.mg/kg buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond,VA) every 12 hrs for 48 hrs.

IVIS optical Imaging

Low-light imaging was performed using an IVIS Kinetic Imaging System consisting of a cooled integrating CCD camera mounted on a light-tight specimen chamber, and controlled by computer. Bioluminescent output was acquired in complete darkness, and the data represented as pseudocolor images indicating light intensity superimposed over the grayscale reference images. Using Living Image analysis software (v 4.0, Xenogen Corporation), light output from specified regions of interest (ROI) was quantified as the total flux, or total number of photons emitted per second. Prior to imaging, animals are injected IP with Redi-inject Luciferin K salt (Calper) at 150mg/kg and were anesthetized using 2% isoflourine while imaging. 10-15 mins after luciferin injection, imaging data were collected with 3min exposure times. Animal protocols were all approved by animal care and use.

Statistical analysis

Experiments were repeated at least 3 times with duplicate or triplicate samples for each condition. Data from representative experiments are presented. Similar trends were seen in multiple trials.

CHAPTER VII

***In Vivo* Grafting of Human Hepatic Progenitors into Immunocompromised Murine Hosts**

Rachael Turner, Elaine Wauthier, David Gerber, and Lola M Reid

Abstract

Patients with severe liver disease can undergo liver failure, remedied by liver transplantation. Alternative therapies include the transplant of donor cell suspensions with hopes of restoring liver functions in the recipient. Current methods of cell transplantation are performed via vascular routes that yield inefficient engraftment, distribution of cells to non-target sites, and emboli formation. This study investigates alternative methods focused on grafting technologies in which stem/progenitor cells are embedded in a complex of hyaluronans mixed with other extracellular matrix components, hormones and soluble signals, and then grafted into the tissue, localizing the cells to the target organ. The chemistry of the graft materials and the choice of specific cell populations used were designed to mimic the stem cell niche, increasing the success of the transplant to survive, expand and regenerate tissue. Therapies were compared to traditional cell suspension treatments in athymic nude mouse models. It was found that grafting methods improved engraftment of hHpSCs into the livers of the hosts, minimizing their loss to other tissues. *In vivo* luminescent imaging of luciferin-expressing transplanted cells showed that cells grafted into the liver using

hyaluronan hydrogels remained in the liver tissue, whereas cells injected via suspension dispersed throughout the liver and to ectopic sites. Human albumin in the serum showed increased production in injury models due to the feedback signaling in the liver, as well as an increase in production from grafted cells. Histology staining of human albumin showed large masses of transplanted cells when grafted in the host liver, as opposed to smaller single cell groups using cell suspension. Grafting of hepatic stem cells in hyaluronan hydrogels localized cells specifically to the target liver tissue, providing an efficient method for cell transplantation for use in liver regeneration.

Introduction

Patients with severe liver disease can undergo liver failure, remedied in extreme cases by orthotopic liver transplantation (OLT). Due to the high cost of transplant treatment, lack of availability of healthy liver donors, and highly expensive liver transplantation treatment, many alternative therapies are being investigated and include the transplantation of donor cell suspensions, with hopes of restoring liver functions in the recipient patients [135, 244]. Hepatocyte transplantation is emerging as alternative bridge support until a healthy donor is arranged [245, 246] However, mature hepatocytes have short-term survival and lack the proliferation capacity of progenitor cells both *in vitro* and *in vivo*. The trials of liver cell therapies to date have made use of mature liver cells and with clinical improvements in patients but with significant problems due to inefficient engraftment (only 20-30% of transplanted cells), distribution of most cells to ectopic sites, emboli formation, immunological rejection, and transient effects of transplanted cells.

Human hepatic stem cells (hHpSCs) are multipotent stem cells located within ductal plates of fetal livers and the canals of Hering of adult livers [19]. The hHpSCs range in size from 7-10 μm in diameter and have a high nucleus to cytoplasmic ratio [18]. They are tolerant of ischemia, and, surprisingly, can be found as viable cells in cadaveric livers for up to 6 days after asystolic death [22, 24]. The hHpSCs form colonies in culture capable of self-replication [25] and of differentiation to mature cells in culture and *in vivo* [18, 111]. They constitute ~0.5-2% of the parenchyma of livers of all donor ages [18]. Their known antigenic profile comprises epithelial cell adhesion molecule (EpCAM), neural cell adhesion molecule (NCAM), cytokeratins (CK) 8/18/19, hedgehog proteins (Sonic and Indian), claudin 3, weak (if any) expression of albumin or MHC antigens, and no expression of α -fetoprotein (AFP),

intercellular adhesion molecule (ICAM-1), P450s, markers for hemopoietic cells (e.g. CD34, 38, 45, 90, or glycophorin), endothelial cells (e.g. VEGFr, CD31, von Willebrand factor) or mesenchymal cells (e.g. CD146, desmin, vitamin A, CD105) [12, 18, 30, 31]. The hHpSCs have pumps such as MDR1 that eliminate xenobiotics. In addition, they express telomerase mRNA and have telomerase protein entirely localized within the nucleus. The hHpSCs are readily isolated by immunoselection for cells doubly positive for [EpCAM + NCAM+] from livers of all donor ages. They expand *ex vivo* if in a serum-free medium, Kubota's Medium, designed for endodermal progenitor cell populations [247]. If transplanted into immunocompromised hosts, they yield mature liver tissue. Distinct culture conditions can be used to drive them selectively towards either mature hepatocytes or cholangiocytes [26].

The lineages of maturing cells within the liver are associated with specific microenvironments of extracellular matrix and soluble signals constituting paracrine signals [248]. The matrix chemistry found in stem cell niches is similar to that found in embryos and includes type III and IV collagens, hyaluronans, and laminins [26]. This matrix/soluble signal combination is optimal for survival and expansion of the isolated hepatic stem/progenitors.

The potential of purified human hepatic stem cells (hHpSCs) for differentiation was previously tested *in vivo* using scid/NOD mice, and within 48 hours of inoculation, the transplanted EpCAM+ cells formed liver-like tissue structures that persisted for weeks, but that would expand dramatically if the animals were treated with carbon tetrachloride, CCL₄, to induce liver injury and, thereby, reduce or eliminate the feedback loop signal(s) [249].

Transplantation of hHpSCs into a portal vein results in less than 5% remaining in the liver, but if via the hepatic artery, results in up to 40% of the cells engrafting [250]. The clinical trials with EpCAM+ cells from fetal livers (both hHpSCs and hepatoblasts, hHBs)

have revealed no evidence of emboli formation and no need for immunosuppression, and provided increased function in all transplanted patients [250]. The cells that distribute ectopically have now been found to survive for months (as described in chapter IV) and are able to form liver tissue in some ectopic sites such as lymph nodes [251]. These findings indicate that ectopic distribution of transplanted cells is a serious concern clinically.

This study investigates alternative methods focused on grafting technologies in which stem/progenitor cells are embedded in a complex of hyaluronans mixed with other extracellular matrix components and with hormones and soluble signals, and then grafted onto or into the tissue, localizing the therapies to the target organ. The chemistry of the graft materials and the choice of specific cell populations used are designed to mimic the stem cell niche, increasing the success of the transplant to survive, expand and regenerate tissue. Therapies were compared to traditional cell suspension treatments in athymic nude mouse models. It was found that grafting methods improved engraftment of hHpSCs into the livers of the hosts, minimizing their loss to other tissues. Histology staining of human albumin showed large masses of transplanted cells when grafted in the host liver, as well as an increase in serum albumin production, as opposed to smaller single cell groups using cell suspension.

Methods

Liver Sourcing and Processing

Fetal liver tissues were provided by an accredited agency (Advanced Biological Resources, San Francisco, CA) from fetuses between 16-20 weeks gestational age obtained

by elective pregnancy terminations. The research protocol was reviewed and approved by the Institutional Review Board for Human Research Studies at the University of North Carolina at Chapel Hill. Suspensions of fetal human liver cells were prepared as described previously [18, 220]. All processing and cell enrichment procedures were conducted in a cell wash buffer composed of a basal medium (RPMI 1640) supplemented with 0.1% BSA (BSA Fraction V; Sigma-Aldrich), insulin and iron-saturated transferrin (both at 5 ug/ml; Sigma-Aldrich), trace elements (300 pM selenious acid and 50 pM ZnSO₄), and antibiotics (AAS; Invitrogen). Liver tissue was subdivided into 3-ml fragments (total volume ranged from 2 – 12 ml) for digestion in 25 ml of cell wash buffer containing type IV collagenase and deoxyribonuclease (both at 6 mg per ml; Sigma- Aldrich) at 32°C with frequent agitation for 15–20 min. This resulted in a homogeneous suspension of cell aggregates that were passed through a 40-gauge mesh and spun at 1,200 RPM for 5 min before resuspension in cell wash solution. Erythrocytes were eliminated by either slow-speed centrifugation or by treating suspensions with anti-human red blood cell antibodies (1:5,000 dilution; Rockland) for 15 min, followed by LowTox Guinea Pig complement (1:3,000 dilution; Cedarlane Labs) for 10 min, both at 37°C. Estimated cell viability by Trypan blue exclusion was routinely >95% [33, 184, 221].

Hepatic Stem Cell Culture Conditions

For all cultures, Kubota's Medium (KM), specially designed for the expansion of hepatic progenitors, was used. KM was developed originally for rodent hepatoblasts [222] and subsequently found effective for hHpSCs and hHBs [25, 37, 184, 221], for biliary tree stem/progenitors (Cardinale/Wang et al, submitted) and for pancreas (Wang et al, manuscript

in preparation). KM consists of any basal medium, here being RPMI 1640 (Gibco /Invitrogen, Carlsbad, CA) with no copper, low calcium below 0.5 mM (here being 0.3 mM), trace elements (zinc--10⁻¹²M and selenium—10⁻⁹M), insulin (5 µg/ml), transferrin/fe (5 µg/ml), high density lipoprotein (10 µg/ml) and a mixture of free fatty acids bound to bovine serum albumin. Detailed methods for its preparation are given in a methods review [33].

HA matrix Preparation

All hylauronan materials are commercially available from Glycosan Biosciences (Salt Lake City, Utah)[now part of Biotime, Alameda, CA], and consist of thiol-modified carboxymethyl HA (or CMHA-S), a chemically modified HA derivative with disulfide bridges for cross-linking. The cross-linking to form a gel is initiated by a PEGDA crosslinker, enabling the hydrogels to be formed in the desired container [219, 223-226]. The level of crosslinking activity and stiffness of the hydrogel can be regulated by the amount of PEGDA added. The hydrogel substrata was constructed by dissolving dry reagents in KM to give a 2.0% solution (weight/volume) for the HA gels and the PEGDA crosslinker was dissolved in KM to give a 4.0% weight/volume solution. Samples were then allowed to incubate in a 37° C water bath and allowed to completely dissolve. Collagen III and laminin samples were obtained from Sigma (St. Louis, MO) and were used at a concentration of 1.0mg/ml. A ratio of 1:4 was applied to blend the crosslinker and hydrogels. At the end of the experiment, hydrogels were dissolved easily using dithiothreitol reducing agents with hyaluronidase without damaging the cells.

Mice.

Athymic nude, male mice, aged 8-12weeks, were bred in house bred in-house at the UNC Animal Care Facility. Animals received care according to the Division of Laboratory Animal Medicine, UNC-CH guidelines, ones approved by AALAC. All animal protocols were approved by IACUC with regard to animal care and use.

In vivo engraftment

After cell isolation, freshly isolated hepatic progenitor cells were infected for 4 hrs at 37°C with a luciferase-expressing adenoviral vector at 50 POI (Vector Biolabs, Philadelphia, PA). Mice (8-12 weeks) were anesthetized using ketamine (90-120mg/kg, Bioniche Pharma, Lake Forrest IL), and xylazine (10mg/kg, Akorn, Decatur, IL). Survival surgery was performed, opening the abdomen and slowly injecting 1.5×10^6 cells directly into the liver lobe, via cell suspension or grafted using HyStem Hyaluronans (Glycosan, Alameda, CA) crosslinked with Poly (Ethylene Glycol)-Diacrylate (PEG-DA) intrahepatically into the front liver lobe. The incision site was closed, and animals were given 01.mg/kg buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond,VA) every 12 hrs for 48 hrs. Past studies [184] have indicated that giving the mice the cells first and then establishing liver failure results in survival of all the transplanted mice. For liver injury models, a one-time dose of carbon tetrachloride (CCL4, Sigma-Aldrich, St Louis, MO) was administered IP at 0.6 ul/g.

Optical Imaging

Low-light imaging was performed using an IVIS Lumina Imaging System that consists of a cooled integrating CCD camera mounted on a light-tight specimen chamber, and controlled by computer. Bioluminescent output was then acquired in complete darkness, and the data are represented as pseudocolor images indicating light intensity superimposed over the grayscale reference images. Using Living Image analysis software (v 4.0, Xenogen Corporation), light output from specified regions of interest (ROI) is quantified as the total flux, or total number of photons emitted per second. Prior to imaging, animals were injected IP with Redi-inject Luciferin K salt (calper) at 150mg/kg and were anesthetized using 2% isoflourine while imaging. At 10-15 mins after luciferin injection, imaging data was collected with 3 min exposure times.

Analysis of secreted protein production

Concentration levels of secreted human albumin in mouse serum at day 7 was assessed to determine the function of the transplanted human hepatic progenitor cells. Albumin production was measured by ELISA using human albumin ELISA quantitation sets with respect to human reference serum standards from the manufacturer (Bethyl Laboratories, Montgomery, TX) in terms of horseradish peroxidase (HRP) -conjugated fluoroprobe levels (detection antibody against albumin) by colorimetric absorbance at 450 nm. Assays were measured individually with a cytofluor Spectramax 250 multi-well plate reader (Molecular Devices, Sunnyvale, CA).

Histology

At day 7, tissue samples were removed from mice and fixed 2 days in 4% PFA and stored in 70% Ethanol. Samples were submitted to the CGIBD Histology Core Laboratory at UNC Chapel Hill for paraffin imbedding and serial sectioning at 5 μ m section thickness. Staining was performed by the Histology Research Core Facility at UNC Chapel Hill. Sections were deparaffinized with xylene and rehydrated with decreasing alcohol series. Quenching of endogenous peroxidase activity was performed by first incubating in H₂O₂/H₂O and then blocking with goat serum. The primary antibody for human albumin anti-rabbit (Abcam, Ab2406) was diluted at 1:1000. Biotinylated goat anti-rabbit secondary antibody solution was then applied at 1:500 dilution, followed by VECTASTAIN Elite ABC Reagent at 1:500 (pk6100, Vector Laboratories, Burlingame, CA). Sections were analyzed using an Olympus IX70 Inverted Fluorescence Microscope equipped with a Hg/Xe arc lamp for epi-illumination and an Olympus DP72 Digital Camera controlled with cellSens™ Digital Imaging Software for image acquisition.

Results

To test efficiency of cell localization using grafting strategies, optical imaging was used to detect the bioluminescent signal produced from Luciferin-infected cells localized within the mice. **Figure 19** shows the total flux detected in animals at sites where cells were injected with and without grafting methods. In both healthy and injury models, there was no significant difference between the signal produced by grafted cells versus suspension cells. However, there was a considerable difference in the consistency of cells located within the abdomen of grafted models, as the standard error in suspended cells is much greater. The

location of luminescent-producing cells can be seen in **Figure 20**. Cells grafted to the liver using hyaluronans were specifically localized to the injected liver tissue. Cells that were injected intrahepatically via suspension were observed as spread throughout the abdomen.

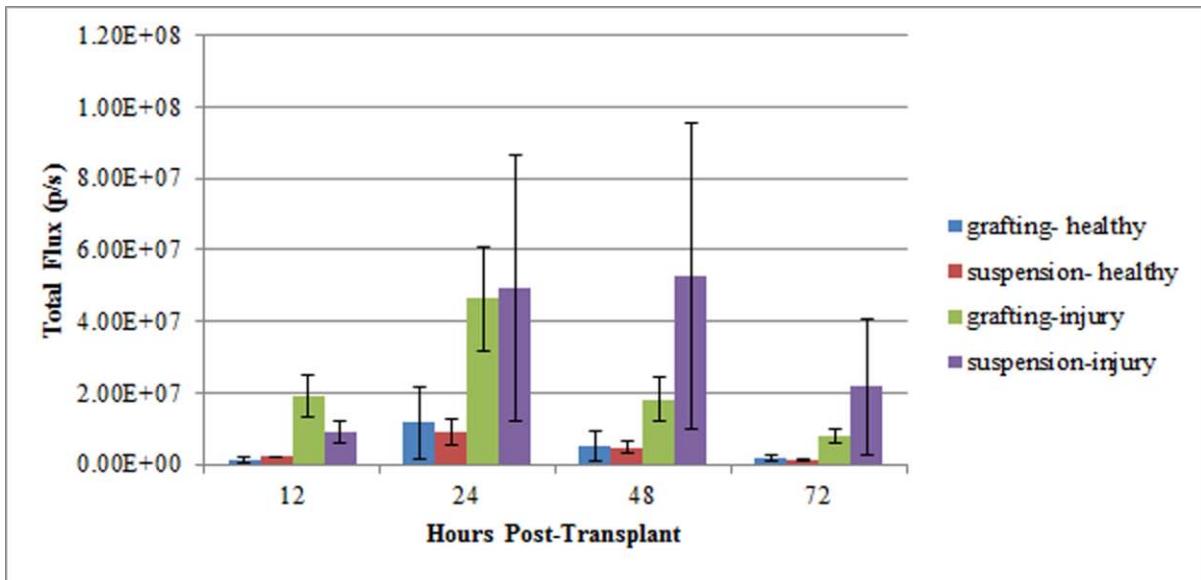


Figure 19: Total flux signal captured by luciferin expressing transplanted cells grafted with hyaluronans versus injected as a cell suspension in healthy and CCl4 induced liver injury models. Flux readings are normalized to control animals receiving no cell transplant.

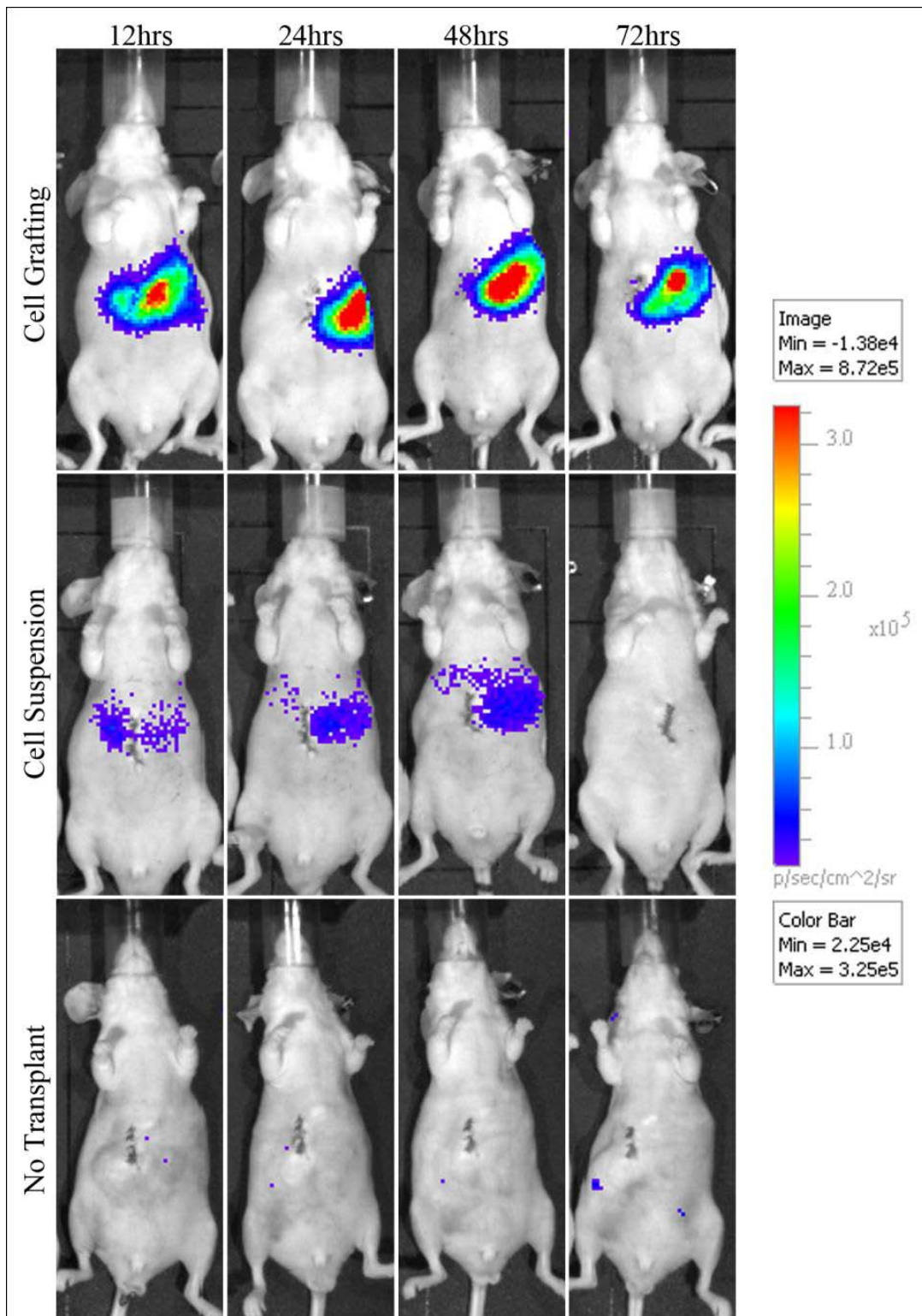


Figure 20: in vivo real time imaging of luminescent signal produced by luciferin-producing cells both grafted with hyaluronans versus injected as a cell suspension.

At day 7, blood sampling was taken, and tissues were removed and fixed for histology. Human albumin was measured in the murine blood samples, and can be seen in **Figure 21**. A slight increase in serum albumin was seen in the injury model versus healthy model. Grafting methods also showed an increase when compared to the results from cell suspensions. However, neither of these trends were shown to be significant.

Tissue histology at day 7 in CCl₄ treated mice can be seen in **Figure 23**. Tissue was stained for human albumin, marking transplanted cells brown in the host tissue. Cells transplanted via grafting methods using HA are in panels A-D. Here, cells were grouped and maintained large cell masses of transplanted cells within the host cells. Cells transplanted via cell suspension (panels E-H), resulted in small aggregates dispersed throughout the liver. Staining controls given in **Figure 22**.

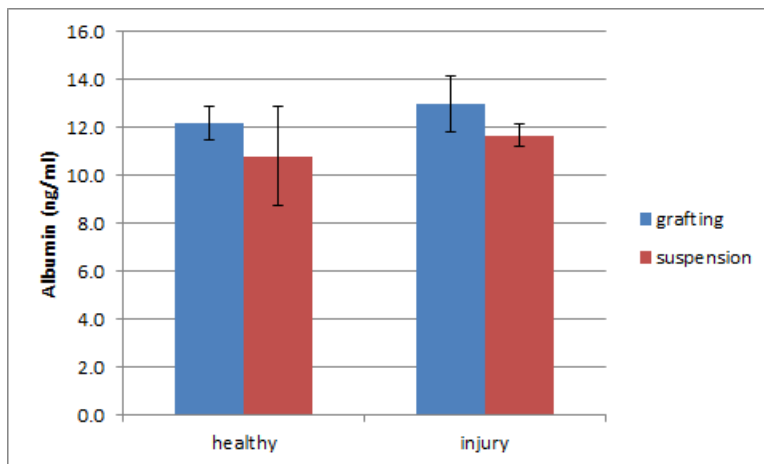


Figure 21: Serum human albumin at day 7 post-transplantation in grafted versus cell suspension in both healthy and CCl₄ liver injury models.

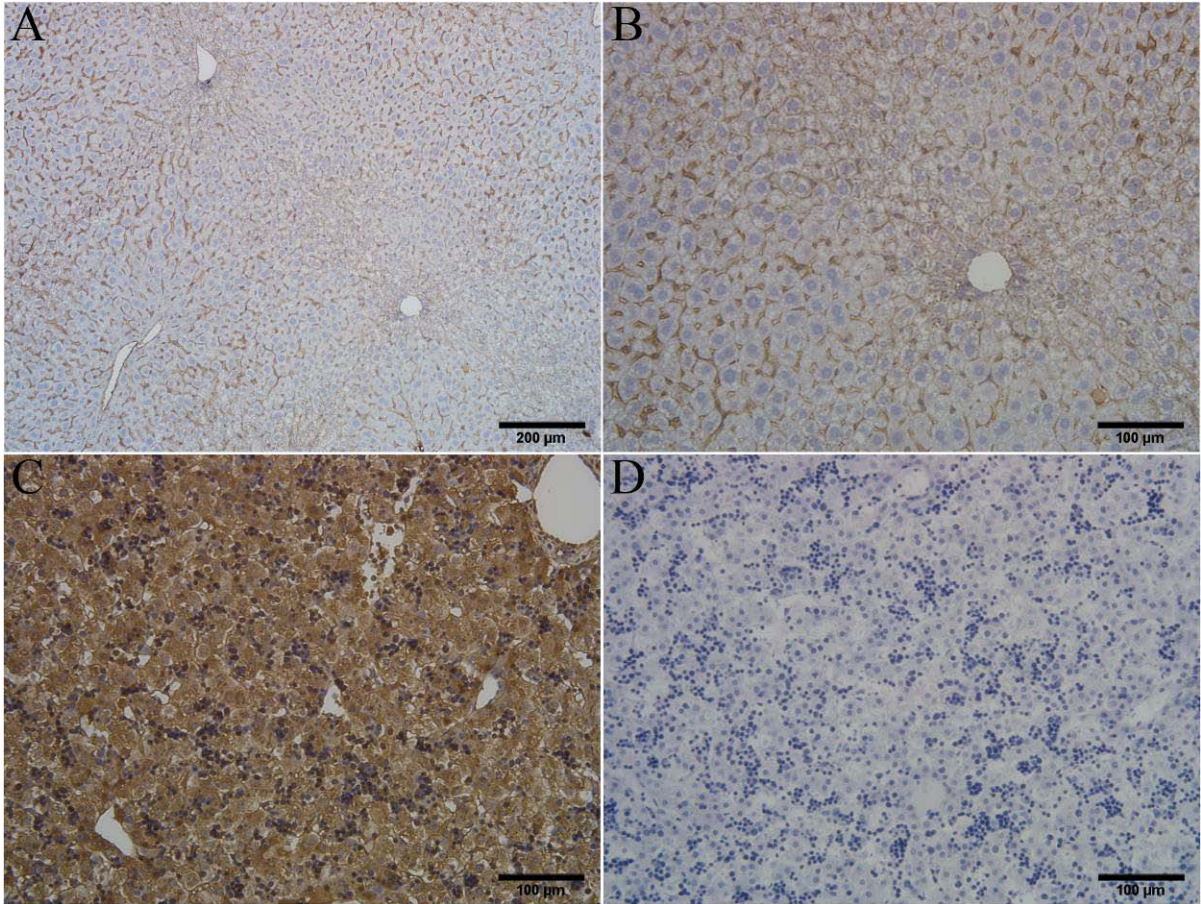


Figure 22: Controls for Histology of Human Transplanted Cells. A) and B) Control- no transplant. C) positive human fetal liver for human albumin and D) human fetal liver, no primary antibody staining.

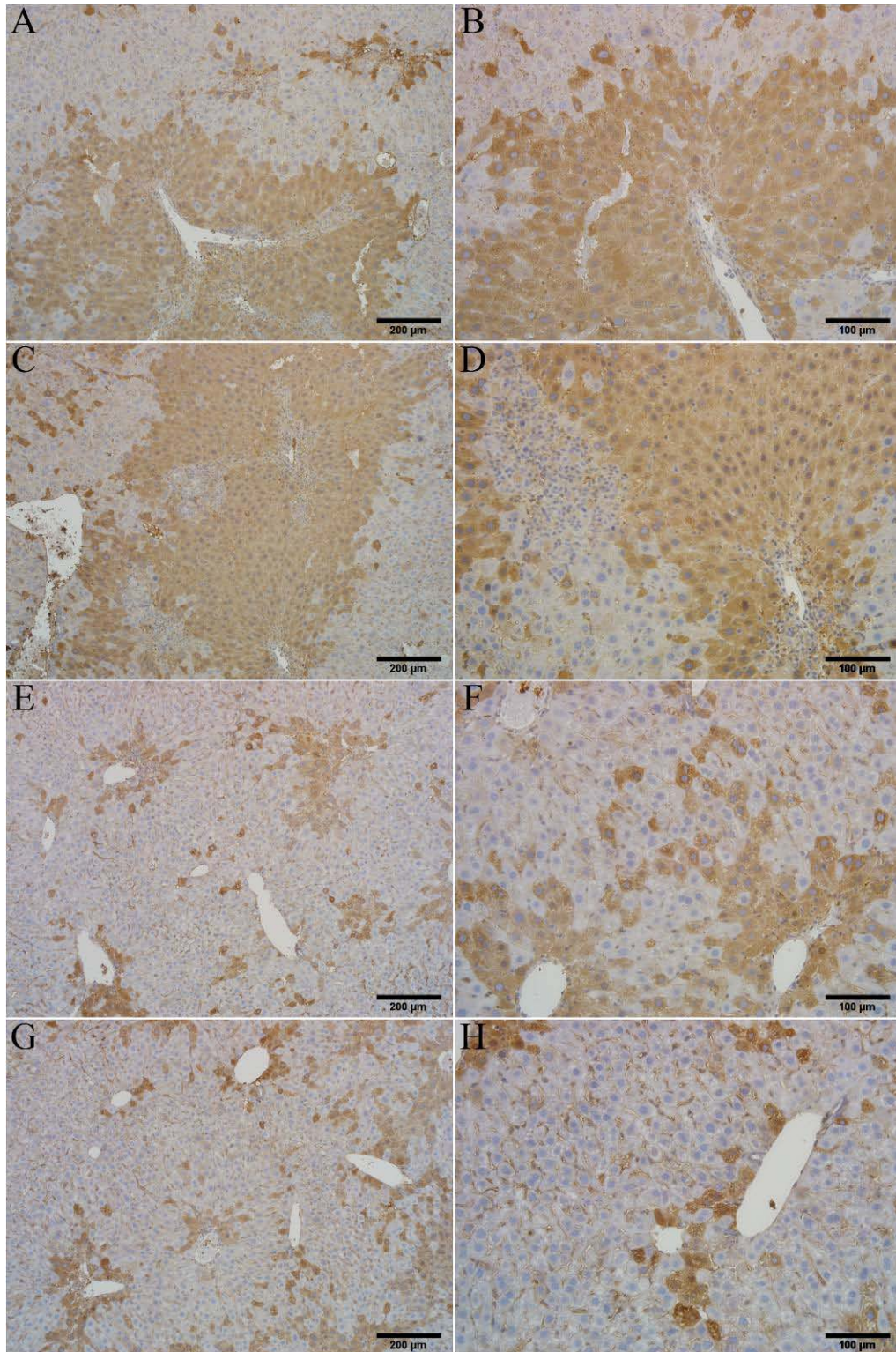


Figure 23: Histology of Human Transplanted Cells. Transplanted human hepatic progenitor cells stain positive for human albumin. Day 7 post-transplant. A-D) Grafting using Hyaluronan Hydrogels. E-H) Cell suspension.

Discussion

In vivo imaging data shows that cells transplanted with and without grafting techniques produced similar signal intensities, meaning that the same number of cells remained within the animals. However, unlike the grafting therapies where cells remained located within the liver tissue, cell suspensions were dispersed throughout the body. This is similar to other findings, where cells injected into ectopic sites like the spleen lead both to engraftment within the liver but always also spread into secondary vascular beds such as those in the lungs [252, 253], and have also been found to form liver tissue in ectopic sites such as lymph nodes [251]. This is a serious concern clinically, and these grafting methods that localize therapies have shown to be a possible solution.

The use of biocompatible matrix materials in cell therapies is not a new concept. Matrix components are the primary determinants of attachment, survival, differentiative responses, cytoskeletal organization, and stabilization of requisite cell surface receptors that prime the cells for responses to specific extracellular signals [33, 141-143]. The selection of matrix components is guided by gradients *in vivo*, that change from the stem cell compartment to the late lineage stage cells. The matrix chemistry changes with maturational stages, with host age, and with disease states [150]. Therefore, graft biomaterials should mimic the matrix chemistry of the particular lineage stages desired for the graft. The use of hyaluronans as an injectable material for tissue engineering is promising due to its long-lasting effect while maintaining biocompatibility [233] and ability to minimize patient discomfort, lower risk of infection, scar formation, and overall cost [254]. Co-culturing and injecting with a cell suspension allows for solidification *in situ* that can fill any cavity space. Cross-linking methods also maintain the material biocompatibility, and its presence in

extensive areas of regenerative or immature tissue areas make it a very attractive injectable material.

Grafting of cells using injectable biomaterials has been shown to be successful for therapies other than the form of liver injury discussed here. Studies involving *in situ* engineered tissue, including studies of injectable Matrigel with embryonic stem cells [255, 256] and fibrin with skeletal myoblasts [257, 258] have shown restoration of cardiac function and geometry after cardiac injury. Injectable materials solidify *in vivo* and retain the geometry of the injured tissue. In all of these studies, these materials have not only been shown to provide a microenvironment for the cells post-injection, but also may specifically influence the microenvironment to prevent disease progression and induce increased regeneration within the tissue.

The grafting methods presented in this study showed that cells remain localized to the liver tissue after injection. *In vivo* luminescent imaging looked at the luminescent signal produced within the abdominal region of interest. A large signal was measured in both suspension and grafting methods (**Figure 19**), showing that cells are in fact present within the animal in both cases. Since the animals are immunocompromised, it is not surprising that all cells are present. However, images (**Figure 20**) better explain the exact location of the cells. While a signal is produced by both transplanted methods, it is clear that the cells are specifically localized to the liver lobe in grafting but not when injected via a vascular route. Additionally, the small standard error of total flux produced in grafting (compared to the large error seen in cell suspension) shows the consistent transplantation of cells within the tissue. Histology of host tissue at day 7 further exemplifies the consistent localization of transplanted hepatic stem cells if via a grafting strategy. In HA grafts, cells form large

masses of cells, remaining localized to the liver tissue where injected. The opposite is seen in cell suspension injections, where cells that manage to stay within the liver tissue are present only in smaller, more dispersed groups. The combination of *in vivo* imaging and tissue histology gives a macro and micro image of transplanted cell location within the animals, and wholly supports the need for grafting methods as strategies for cell transplant therapies.

Liver injury is necessary in promoting liver cell growth. Selective loss of pericentral cells with toxic injury results in muting of the feedback loop signaling and secondarily activates rapid cell division of early lineage stage cells [23, 120]. In response, periportal cells undergo rapid hyperplastic growth (complete cell division) followed by differentiation. These phenomena, the classic “oval cell response” in rodents and the “ductular reactions” seen in human massive hepatic necrosis (e.g. acetaminophen toxicity, acute hepatotropic viral infection), have long been recognized to involve extensive expansion of the stem/progenitor cell populations [23]. Carbon tetrachloride exposure results in loss of late lineage stage cells, eliciting chronic regenerative responses. As seen in the production of human serum albumin, an increased amount of albumin is produced in the injury model mice.

This study investigated alternative methods focused on grafting technologies in which stem/progenitor cells are embedded in a complex of hyaluronans mixed with other extracellular matrix components and with hormones and soluble signals, and then grafted onto or into the tissue, localizing the therapies to the target organ. Graft conditions can also be modified to have factors to improve vascularization, expansion and/or differentiation. The chemistry of the graft materials and the choice of specific cell populations used are designed to mimic the stem cell niche, increasing the success of the transplant to survive, expand and regenerate tissue.

CHAPTER VIII

Conclusions

Conclusions

This body of work addresses key problems associated with liver regeneration using tissue engineering themes. By utilizing the proliferative capacity of the stem cell population, along with its associated matrix, grafting methods were developed by embedding the cells into a microenvironment that mimics the liver's stem cell niche. This combination of ECM with the cells was proven successful in cryopreservation techniques, *ex vivo* culture, and *in vivo* grafting methods. The techniques developed as part of this dissertation have improved current alternative methods available for the treatment of liver failure, and can easily be translated and applied.

The supply of human hepatic cells is often limited and dependent on the availability of liver tissues from resections and rejected organs from organ donation programs. The first aim of this dissertation was to improve the methods for cryopreservation and long term storage of cells, vital in facilitating full use of the cells and thereby optimizing this scarce resource. Freshly isolated human hepatic progenitor cells were isolated from fetal liver tissue and cryopreserved in either KM culture medium or CS10 cryopreservation medium, and utilized with or without small amounts of hyaluronans (0.05 or 0.10%). Samples were frozen using a controlled rate freezing program. Post-thaw cell viability, attachment, and colony growth were measured. Gene and protein expression of Integrin β 1, Integrin β 4, E-Cadherin,

and CD-44 were evaluated by qRT-PCR and immunohistochemistry analysis. Results showed that cells thawed with CS10 isotonic medium supplemented with small amounts of HA (0.05 or 0.10%) improved cell attachment and colony formation due at least in part to improved expression of key cell adhesion factors in the stem cells and progenitor cell populations. CS10 medium, supplemented with KM supplements, also showed improved attachment when compared to other medium conditions without gel. Future studies may include this medium combination with the addition of hyaluronans, a freezing condition not previously considered. This study provides successful methods in primary hepatic progenitor cell cryopreservation, and should be implemented to freezing methods for more efficient banking methods in both research and potential therapy applications.

The second development that has importance in liver cell therapies is a method of culturing hHpSCs and hHBs in a three-dimensional (3-D) environment. It was hypothesized that hyaluronan hydrogels, present in all stem cell niches including those for liver, would be a successful base material for maintaining the hepatic stem cell phenotype. In this study, cell aggregates formed within the HA hydrogels, remained viable, and demonstrated a stable phenotype for hepatic stem cells for at least 3 weeks of culturing. The phenotype consisted of stable co-expression of EpCAM, NCAM, and albumin, and with maintenance of hepatic function through albumin and transferrin production and urea synthesis. Hyaluronans, combined with collagen III and laminin, maintained hepatic function and further expressed stem cell characteristics, proving to be a suitable three-dimensional scaffolding for hHpSCs.

The last aim investigated in this study was the use of injectable hyaluronan grafts as a method of cell transplantation *in vivo*. Current methods for cell therapies introduce donor cells into hosts via suspension either directly into tissue or via a vascular route, resulting in

inefficient engraftment of cells in the appropriate location, engraftment of cells at sites other than the liver (e.g. lung), as well as emboli formation. It was hypothesized that grafting methods, combining cells within an appropriate matrix and hormonal environment to provide an optimal starting environment for survival, expansion and vascularization. The chemistry of the graft materials and the choice of specific cell populations used was a result of the previous aim, and was designed to mimic the stem cell niche, increasing the success of the cell survival, expansion, and regeneration of tissue. *In vivo* luminescent imaging of luciferin-expressing transplanted cells showed that cells grafted into the liver using hyaluronan hydrogels remained in the liver tissue, while cells injected via suspension dispersed throughout the liver. Histology data further supported these findings, where HA grafts facilitated the formation of large masses of cells, remaining localized to the liver tissue where injected. The opposite was seen in cell suspension injections, where cells were present only in smaller, less localized groups. Human albumin in the serum showed increased production in injury models due to the feedback signaling in the liver, as well as an increase in production from grafted cells. These studies support the need for grafting methods within cell transplant therapies; grafting technologies developed are preclinical, but expected to be rapidly translatable to therapeutic uses.

Recommendations for Future Research

This research explored two key areas to improve liver therapies; the cell sourcing and the grafting of cell therapies. Further studies in cryopreservation involve investigating the combination of media supplements known to be essential in the culturing of hepatic stem cells with CS10 freezing medium, in combination with hyaluronan supplements. This

condition was not previously investigated and may increase viability and attachment. However, the study still provided successful methods in primary hepatic progenitor cell cryopreservation, and these conditions should be implemented to freezing methods for more efficient banking in both research and potential therapy applications.

Moving forward, a final and logical step would be the grafting of cryopreserved cells. Stem cells intended for liver therapies can be frozen at the time of isolation. Cells can be banked until needed, then thawed and immediately used in grafting programs.

An additional method of grafting initially discussed in chapter III was the development of patch grafts. Here; grafting can be done external to the tissue, rather than directly injected. A native or synthetic biomaterial can be adhered to the liver surface, then the injectable hydrogel grafting material can be implanted in the pocket made by the patch. Collaborations have been made with Dr. David Kaplan at Tufts University (Boston, MA) and the Tissue Engineering Research Center. Together with members of the center, we have designed and constructed porous patches made of biocompatible silk [264, 265]. The patches are 500 μm pore size around 95~99% porosity, with a non-porous outside surface. The hollow “hat” patch is 6.5mm in diameter, with a 2-3mm ridge border for adhering to the tissue (**Figure 24**). This external patch would allow for larger volumes of cells, and does not injure the tissue like intrahepatic injections, allowing for broader therapeutic applications.



Figure 24: electrospun silk patch for external patch grafting.

Lastly, the obvious and more long-term goals are the translation to clinically relevant therapies. All materials used in this dissertation were chosen for their ease and transition to clinical therapies. The use of cryo-cell banking is an important part in the sourcing of adequate cell numbers for human cell therapies. The rapid translation to therapeutic uses in patients will benefit by providing an alternative treatment to transplants that may regenerate lost tissue.

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