DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS ALONG A HEPATOCYTE LINEAGE

By

ERIC I. NOVIK

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Biomedical Engineering

written under the direction of

Dr. Martin Yarmush

and approved by

_____

_____

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2007

ABSTRACT OF THE DISSERTATION

Differentiation of Mouse Embryonic Stem Cells Along a Hepatocyte Lineage
By ERIC I. NOVIK

Dissertation Director:

Dr. Martin Yarmush, MD, PhD

The development of implantable engineered liver tissue constructs, ex vivo hepatocyte based therapeutic devices and drug discovery studies are limited by an inadequate hepatocyte cell source. Embryonic stem (ES) cells, characterized by their self-renewing and multi-lineage differentiating capabilities, represent a promising mature cell source required for these applications. Previous research has utilized embryoid body (EB) formation in both guided, through extracellular matrix and growth factor supplementation, and unguided, or spontaneous, differentiation to generate hepatocyte like cells. However, these characterizations have been limited to only one or several lineage specific protein or gene expression patterns. In addition, there have been few reports of long term propagation or characterization of long term function for ES cell derived hepatocyte precursors. In this thesis, we have implemented a platform for the long term propagation and augmentation of functional hepatocytes generated from murine ES cell sources. We first utilize a controlled, reproducible, EB mediated differentiation system to characterize efficiency of hepatocyte lineage commitment in four parallel culture configurations. These studies have shown that, EB mediated stem cell differentiation spontaneously yield populations of hepatocyte lineage cells expressing mature hepatocyte markers such as albumin (ALB) and Cytokeratin 18 (CK-18). We then used secondary culture configurations to study the effects of collagen sandwich culture and Oncostatin-M (OSM) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) supplementation of EB derived hepatocyte-lineage cell function. The results of these studies suggest that SNAP, independent of the collagen supplementation, maintains the highest levels of ALB expression, however mature liver specific CK-18 is only expressed in the presence of both gel sandwich culture supplemented with SNAP. In addition, albumin secretion and Cytochrome P450 detoxification studies indicated that this condition was the best for the augmentation of hepatocyte-like function. Maintenance and augmentation of hepatocyte-like cells isolated from heterogeneous EB cell populations will be a critical step in generating large numbers of functional differentiated cells for therapeutic use.

ACKNOWLEDGMENTS

As I approach the end of my time here at Rutgers I often find myself reminiscing about the last six years and the Route 66 of life that has led me to this point. The time has been one of great personal growth both intellectually and philosophically. My part in this growth is actually not as large as I would like to think. It was facilitated by the countless number of individuals who have in some way shaped the individual you and I (looking in the mirror) see today. I will not mention everyone here but will always know who they are and the role they played in my life, even if they may not, and for this I offer them my thanks.

I would like to mention some of my closest friends and lab mates first. These are the people I spent almost every day and sometimes night of the past six years with. Natesh Pareshurama, Eric Wallenstien, Nripen Sharma and Tim Maguire make up the core of the Yarmush/Schloss team. If two minds are better than one, then these four are better than twenty. They have been my sounding board for experimental ideas, golf partners and excellent friends and for this I thank them. Recently there have been a few additions to the lab group, Lu Lu Li, Kevin Nikitczuk and Jean-Pierre Dolé and they have also come to be good friends and constructive critics of my work. I know that we will remain friends for many years to come.

To my mentor and advisor, Dr. Martin Yarmush (Maish) I would like to say thank you. Maish's vision for development both in the science arena, and in the construction of the Rutgers BME department is inspiring. Many times since our first Whitaker site visit, in which I was indoctrinated into the world of Maish, I have had the honor of playing an integral role in a variety of educational projects. For these opportunities I thank him most, since they have taught me new ways to think about research, and have given me confidence to venture into new areas that prove a worthwhile learning experience. Maish has allowed me to have freedom of thought and allowed me to define my own research path qualities, which are usually difficult to find.

Dr. Rene Schloss has had a major impact on all aspects of my life. I know she will not like the spotlight so I won't go too deep. Rene has always made it a point to go out of her way to help us feel comfortable with our writing and presentations even if that meant inviting us to her home on a Sunday afternoon. She is also

my personal therapist although she might not know it. No matter how frustrated or annoyed I have been over the years for both personal and academic reasons, all I had to do was speak to Rene for ten minutes. After our talks I felt renewed and calm, able to do what I must with a better attitude. She has provided thought provoking discussions on our research and helped to truly define the stem cell research program within the BME department. I know that she will be a life long mentor, teacher and friend.

I am also grateful to the members of my thesis committee: Henrik Pedersen, Arno Tilles, and Charles Roth. I have known you for most of my graduate career, and you have become true mentors. Thank you for taking the time to help me in my research endeavors and thesis studies.

And now I move on to my family. Those of you that know me well know the close relationship I have with my family. I cannot thank my own parents, Michael and Julia, and my sister, Raissa, enough for their love and interest in my work. They are truly special. My parents taught me, leading by example, how to work hard, sometimes day and night, to achieve your goals. If not for their tireless efforts and encouragement, I would be a used car salesman. I once asked them how I could ever repay them for what they have done for me, which is more than I could ever describe here, and their response was “Do the same for your children”. They are the most unselfish people I have ever met. My sister is my secret keeper and tennis partner; the best in the world. I also wish my sister the best of luck in her pursuit of her future degree in Psychology. Alex Ifraimov, Jeff Barminko, Ben Mushailov are not only family but friends. It is said “You don’t choose family but you can choose your friends”. I am glad I chose them.

Most of all I would like to thank my wife, Aleksandra. We got married after my first year in graduate school so you will understand what she has had to put up with for the last four years. She deserves an honorary PhD for dealing with me and the insanity that follows me around. If not for her I would have quit at least ten times. You are definitely my better half. I love you.

Thank You All.

TABLE OF CONTENTS

Title Page

Abstract Page ii

Acknowledgments Page iii

Table of Contents Page v

List of Illustrations Page viii

List of Tables Page x

Chapter 1: Introduction

1.1 Background Page 1

1.2 The Liver: Development and adult function Page 2

1.3 Extracorporeal Bioartificial Liver Devices Page 3

1.4 Primary Hepatocytes Page 4

1.5 Hepatocyte Progenitors Page 6

1.6 Embryonic Stem Cells Page 7

1.7 Unresolved Issues Page 12

1.8 Significance Page 13

Chapter 2: Controlled EB Mediated Differentiation to Explore the Efficiency of Hepatocyte Lineage Commitment in Four Parallel Culture Conditions: Insights From Gene Expression Profiles

2.1 Abstract Page 14

2.2 Introduction Page 14

2.3 Materials and Methods

2.3.1 Cell Culture Page 16

2.3.2 EB Size Page 17

2.3.3 *In-Situ* Immunofluorescence Page 17

2.3.4 RNA Preparation for Microarray Analysis Page 18

2.3.5 Microarray Design, Printing and Processing Page 18

2.3.6 Hybridization Page 19

2.3.7 Microarray Data Analysis Page 19

2.3.8 Gene Network Construction Page 20

2.4 Results

2.4.1 Dynamic Studies of Hepatocyte Function Following ES Differentiation Page 20

2.4.2 Microarray Analysis Page 22

2.4.3 Hepatocyte Lineage Gene Expression Page 22

2.4.4 Regulation of Hepatocyte Lineage Commitment Page 23

2.4.5 Acute Phase Gene Expression Page 23

2.4.6 Regulation of Albumin Expression Page 24

2.5 Discussion Page 24

Chapter 3: Augmentation of EB Directed Hepatocyte-Specific Differentiation via Collagen Sandwich and SNAP

3.1 Abstract Page 29

3.2 Introduction Page 29

3.3 Materials and Methods

3.3.1 Cell Culture Page 31

3.3.2 In Situ Indirect Immunofluorescent Cytokeratin-18 and Intracellular Albumin Analysis Page 33

3.3.3 Glycogen Staining Page 34

3.3.4 Sandwich ELISA for Detection of Albumin Secretion Page 34

3.3.5 Urea Secretion Page 34

3.3.6 Measurement of Cytochrome P450 Activity Page 34

3.3.7 Statistical Analysis of Functional Assays Page 35

3.4 Results

3.4.1 Dynamic Studies of Secondary Cultured EB Derived Hepatocyte Lineage Cells Page 36

3.4.2 OSM and SNAP Supplementation Page 37

3.4.3 Collagen Sandwich Culture Page 37

3.4.4 Cytochrome P450 Detoxification Page 38

3.5 Discussion Page 39

Chapter 4: Future Directions

4.1 Synopsis Page 42

4.2 Future Work

4.2.1 Field Consensus Page 42

4.2.2 Scale-up and Maintenance of EB Derived Hepatocyte-Like Cells Page 43

4.2.3 Using EB Differentiation to Derive Alternative Adult Cell Types Page 43

4.2.4 Alternative Stem Cell Sources Page 44

4.3 Conclusions Page 44

Bibliography Page 45

Curriculum Vitae Page 67

LIST OF ILLUSTRATIONS

Figure1.1 Schematic Representation of In-Vivo Lineage Formation. Page 49

Figure1.2 Schematic Representation of Liver/Hepatocyte Structure. Page 49

Figure 1.3 Bioreactor Designs Page 50

Figure 2.1 Schematic Representation of Hanging Drop Protocol. Page 50

Figure 2.2 EB Size and Morphology Characterization. Page 51

Figure 2.3 20x Images of Phase Contrast and In-Situ Albumin Staining on Day 17 of Differentiation. Page 52

Figure 2.4 Intracellular Albumin Expression. Page 53

Figure 2.5 40x Images of Phase Contrast, In-Situ Glycogen Staining on Day 17 of Differentiation. Page 54

Figure 2.6 Pathway Assist Generated Pathways. Page 55

Figure 3.1. Non Supplemented Secondary Culture Characterization. Page 57

Figure 3.2. OSM and SNAP Supplemented Secondary Culture Characterization. Page 58

Figure 3.3. Collagen Sandwich Secondary Culture Characterization. Page 59

Figure 3.4. **Albumin and Urea Secretion Rates in Sandwich Culture.** Page 60

Figure 3.5. Cellular Morphologies. Page 61

Figure 3.6. Cytochrome P450 Detoxification Page 62

LIST OF TABLES

Table 2.1 Effects of the Four Culture Conditions on Differential Gene Expression. Page 64

Table 2.2 Representative Genes Differentially Expressed in at Least One of the Four Differentiation Conditions. Page 64

Table 2.3 Representative Differentially Expressed Mechanistic Genes. Page 65

Table 2.4 Acute Phase Response Genes. Page 66

Table 2.5: Representative Upregulated Genes of Neuron and Cardiac Tissue Lineages. Page 66

Table 3.1. Function Summary for Supplemented Cultures. Page 66

CHAPTER 1: INTORDUCTION

1.1 Background

The liver is one of the largest and most complex organs in the body. As the metabolic center, it regulates exocrine, endocrine, clotting, and excretory functions as well as organic and cholesterol metabolism. The majority of cells comprising the liver are hepatocytes and are responsible for detoxifying the blood, handling all metabolites, producing plasma proteins such as albumin and clotting factors and producing bile. Another unique property of the liver is its ability to regenerate after partial hepatectomy or injury. Nevertheless, due to inadequate treatment options, acute liver failure is responsible for the death of approximately 30,000 people a year in the U.S. Historically, orthotopic liver transplantation has been the only viable treatment option and more than 6000 transplants were performed in 2006. However, there are not nearly enough donor organs available for the approximately 12,000 individuals waiting for a donor organ. Two thousand patients will die during the first year of their wait (United Network for Organ Sharing Organ Procurement and Transplantation Network). Developing new treatment methods such as extracorporeal bioartificial liver (BAL) devices have been proposed as a means to bridge the gap between supply and demand, providing the patients either more time to wait for a donor liver to become available or to allow enough time for the liver to regenerate.

A BAL requires the incorporation of approximately 10^{10} functional, hepatocyte like cells to sustain liver function. However, locating a cell source that can readily sustain such a large quantity of cells has limited the use of BAL therapy. Researchers have investigated various sources such as porcine hepatocytes, which are readily available, but rejection and mismatch between xenogenic and human function are serious disadvantages. Mature hepatocytes have also been investigated as a cell source, but a limited supply of cells and loss of long term *in-vitro* function makes them an unlikely source for a BAL[1, 2]. The practical application of a BAL in biotechnological applications will require a large number of relatively homogeneous and stable hepatocyte like cells. Advances in pluripotent embryonic stem (ES) cell differentiation technology have generated great interest in utilizing ES cells as a renewable source of functioning hepatocytes and will be the focus of this thesis.

1.2 The Liver: Development and Adult Function

The liver is one of the first and largest organs to develop in the maturing fetus. The fetal liver functions as the site of hematopoiesis which is critical because the fetus relies on its own blood supply for survival. Normal liver development involves numerous stages and is influenced by cytokines as well as cell-matrix interactions. Initially the embryo is comprised of the primitive endoderm and primitive ectoderm. The primitive ectoderm then develops into the three main germ layers: definitive endoderm, ectoderm and mesoderm, while the primitive endoderm develops into the visceral and parietal endoderm, which comprise the embryonic yolk sac. The liver, lung, pancreas, thyroid and gastrointestinal tract all develop from the definitive endoderm. The definitive endoderm develops into the gut tube, at which time different tissues are specified along the axes of the tube. The liver bud arises from the ventral endoderm domain of the foregut, close to the developing cardiac mesoderm. It has been shown that fibroblast growth factor (FGF) signaling [3] from the cardiac mesoderm [4] and bone morphogenic protein (BMP) signaling from mesenchyme cells [5] induce liver bud formation from the definitive endoderm, giving rise to fetal hepatocytes. These cells then proceed through a series of maturation steps which entail proliferation, cellular growth, and functional maturation. These final *in-vivo* steps are induced by various extracellular signals such as: (1) dexamethasone (DEX), which induces albumin production and downregulates alpha-fetal protein production (AFP); (2) transforming growth factor beta (TGFβ), which inhibits hepatocyte proliferation and increases albumin production; (3) oncostatin M (OSM) which induces tight cell-cell contact, necessary for maximum differentiated hepatocyte function, maintenance of albumin production, and upregulation of various other hepatocyte functions.

It is important to distinguish between the primitive and definitive endoderm because the primitive endoderm, specifically the visceral portion, contains cells which express many genes commonly expressed in differentiating liver cells but do not contribute to mature liver development [6, 7]. Only cells of the definitive endoderm contribute to liver development (**Figure 1.2.1**). Understanding the mechanisms governing *in vivo* liver development can provide insights into generating mature hepatocyte like cells to be used in liver therapies.

The adult liver is the largest solid organ in the body. It is a key metabolic and detoxification center, containing parenchymal hepatocytes (70%) and various nonparenchymal cell types such as Ito cells, Kupffer cells, and bile duct cells. In addition, the fact that it is the only organ that is capable of regenerating in its adult form implies the presence of adult stem cells. These cells, termed oval cells, were found in the canals of Hering [8], and are capable of inducing proliferation and differentiation of hepatocytes. From a structural standpoint, the liver is divided into lobules centered on a central draining vein. Each lobule is comprised of polygonal shaped hepatocytes organized into unicellular plates. The unique arrangement of parenchymal cells in the liver allows maximal exchange between the blood and hepatocytes (**Figure 1.2.2**).

Hepatocytes are responsible for most liver functions including: the synthesis of albumin and urea, the metabolism of glucose and fatty acids, bile production, cholesterol production and blood detoxification [9]. The liver is able to detoxify compounds through two mechanisms: Phase I and Phase II biotransformation. Phase I reactions involve cytochrome P450, which is localized in the endoplasmic reticulum of hepatocytes, and create polar metabolites through the oxidation of functional groups on the toxic compounds. In Phase II, the toxic compounds are conjugated with a polar group thereby either detoxifying the compound or making it polar for excretion. However, these functions can not be sustained in *ex vivo* cultures. The functional and structural complexity associated with the liver organ increases the challenges in establishing *ex-vivo* hepatocyte culture systems.

1.3 Extracorporeal Bioartificial Liver Devices

The idea of using a BAL has been extensively explored in existing literature. Such a device could serve to support liver function while the host liver is regenerating and if the liver is no longer capable of regenerating, the device could serve as a bridge to gap the sometimes extensive waiting times between the need for a liver transplant and the availability of a donor liver. A BAL can be compared to a kidney dialysis machine where blood is circulated through a device which removes toxic components and reintroduces the blood into circulation. (**Figure 1.3.1A**) In the liver, this is complicated by the fact that detoxification is cell and not diffusion based, as in the kidney. Therefore, a BAL requires the use of large numbers of hepatocytes to accomplish its detoxification requirements. The major engineering issues in designing a

bioreactor which incorporates live cells are: 1) oxygenation 2) shear stress 3) pressure drop. Current experiments in bioreactor design are designed to mimic physiologic ranges of these parameters.

There are two main bioreactor designs for a BAL: the hollow fiber system and the flat plate system. In the hollow fiber system hepatocyte aggregates, seeded on microcarriers, are placed on the outside of a hollow fiber. Oxygenated plasma is then flowed through the system. However, studies have suggested that hollow fiber reactors are hampered by oxygen transport limitations [10, 11] which are critical for the survival of hepatocytes. In the flat-plate system, hepatocytes are seeded as a monolayer on the bottom surface of a flat plate and placed within a parallel-plate flow chamber. Oxygenated plasma is flowed directly over the cells and an internal membrane oxygenator is added to the reactor for enhanced oxygen diffusion [1]. Although recent studies have made significant strides in efficient bioreactor design there exist significant hurdles to be overcome. One such hurdle is to scale-up a device to include the ~10^{10} hepatocytes which would be required to support a patient. The surface area required to sustain this cell mass is large and requires a novel design. The effects of a grooved, flat plate bioreactor design which can accommodate the large cell mass have been shown to decrease detrimental effects of shear stress while providing adequate oxygenation [12]. (**Figure 1.3.1B**) However, identification of a sustainable cell source is still the major setback in development and implementation of BAL as a treatment for patients suffering from liver ailments.

1.4 Primary Hepatocytes

A well characterized approach for identifying a suitable cell source of hepatocytes has been the optimization of culture conditions to maximize *in vitro* adult primary hepatocyte function. Studies have shown that duration of *in vitro* function depends upon culture conditions such as the type of substrate used, spatial orientation of the cultured cells, addition of growth factors, and the combinatorial effects of these parameters. Many studies have examined the functional response of mature hepatocytes to the physical and chemical properties of culture substrates. In one example, physical culture primary hepatocytes were cultured in a number of collagen and matrigel configurations including monolayer, collagen sandwich, Matrigel sandwich or composite (collagen/Matrigel) sandwich. Collagen sandwich and Matrigel cultures yielded superior and comparable albumin secretion for at least 2 weeks. The data also showed that

hepatocyte polarity could be manipulated by the overall ECM composition independent of the actual morphologies of the cells in different substrates [13].

The effects of growth factor (GF) in addition to extracellular matrix supplementation on the maintenance of hepatocyte function have also been well studied. In one such study, the compliance of Matrigel was altered and the response to the addition of two GFs, epidermal growth factor (EGF) and hepatic growth factor (HGF), was monitored [14]. It was shown that GF supplementation had no effect on hepatocyte function for cells cultured on a rigid substrate. However, compliant matrices augmented the addition of GFs, yielding the highest levels of albumin function. Therefore, the compliance of the ECM can prime the cell for either differentiation on a compliant surface or proliferation on a more rigid substrate. On a compliant substrate, cells were primed to differentiate and hence the addition of GFs led to increased differentiation. These studies are significant in that they decouple the effects of GFs from the effects of compliance on the maintenance of *in vitro* hepatocyte culture.

Other methods to maintain function of isolated hepatocytes include the co-culture of primary hepatocytes with non-parenchymal cells. These co-cultures mimic the cell-cell interactions that are important in all facets of embryonic and adult physiology. It has been shown that when primary rat hepatocytes are cultured with fibroblasts, there is a marked increase in hepatocyte function as compared to hepatocytes cultured alone [15]. In general, systems yielding the most promising results are based upon the aggregation of hepatocytes into spheroids, which markedly increases hepatocyte function. In one system, hepatocyte aggregation was induced by plating hepatocytes on low-density fibronectin [16]. Hepatocytes on this ECM initially attach and remain rounded, and over a few days reassemble into spheroids. Such self-aggregated spheroids show an increase in cytochrome P450 1A1 (CYP1a1) activity as compared to hepatocytes that remain in a monolayer configuration. Hepatocytes with the highest concentration of cell-cell contact, also exhibited the highest level of CYP1a1 activity, thereby reinforcing the idea that morphology and function are correlated. In another study, hepatocyte aggregates and hepatocyte monolayers were cultured in collagen gels [17]. Here again, hepatocyte aggregation occurred with increased function as aggregates expressed an average two-fold increase in urea and albumin production compared to monolayer-plated

cells. The increase in function seen in this system was attributed to an increase in cell-cell contact, as well as maintenance of spheroid morphology. The inherent complexity of such co-cultures and ECM addition cultures creates difficulty in characterization, because although they increase function, they do not sustain function for extended periods of time.

Researchers have tried many culture techniques to prolong isolated hepatocyte function for use in a BAL. To date there are three prevailing hepatocyte culture systems: co-culture, three dimensional networks and aggregate culture. Although these culture systems have been shown to increase and prolong hepatocyte function, the long term function of these cells is generally unstable. There have also been attempts made to immortalize human hepatocytes but function has been poorly characterized. In addition, while all of these studies describe various techniques to boost *in-vitro* hepatocyte function, they do not address the problem of access to primary hepatocytes, as transplantation of the whole liver is much more common than hepatocyte isolation for use in BAL. Xenogenic sources, such as porcine hepatocytes, have been explored as a potential cell source but liver specific function decreases over time and host compatibility is a serious problem that needs to be overcome [2]. Lack of long term function, availability of donor livers and donor to host compatibility limit the ability of isolated hepatocytes to be used in tissue engineered constructs.

1.5 Hepatocyte Progenitors

Liver progenitor cells have been identified in both adult and fetal liver as well as in bone marrow and cord blood. The fact that the liver can regenerate has prompted a variety of research groups to study the role of adult stem or oval cells, located in the canals of Hering, in the liver's response to injury [18-20]. They have been shown to require growth factors such as TGFα, EGF, and HGF for progression through the cell cycle and subsequent differentiation toward mature hepatocytes [21]. Their behavior has been well studied and shown to act like a bi-potential progenitor cell in cases of sever liver injury and mimic hepatoblast differentiation during fetal liver development[22-24]. Recent studies have shown that, in fact, there are two types of pluripotent progenitor cells found in adult livers, oval cells and hepatoblasts. Hepatoblasts are generally found in the fetal liver while oval cells are found in the adult and pediatric livers. Studies have investigated the differentiation capabilities of fetal liver stem cells into mature hepatocytes in the presence

of growth factors and extracellular matrix proteins [25, 26]. However, lack of specific surface markers and low number of cells, 0.3%-0.7% oval cells and <0.1% hepatoblasts, of the total adult liver mass hampers efforts to isolate and study proliferation and differentiation of these cell types [27, 28].

Hematopoietic stem cells (HSCs), isolated from adult bone marrow, have also been induced to differentiate along hepatocyte specific pathways. For example, one experimental system utilized HGF and EGF addition to derive albumin staining polygonal cells from unfractionated bone marrow [29]. Others isolate subpopulations of hematopoietic cells with similar surface markers to oval cells and subsequently differentiate in the presence of growth factors [30]. However, similar to oval and hepatoblast cells, the low number of cells within these subpopulations hampers research efforts. In addition, in these studies proliferative potential, mechanisms of differentiation and many quantitative hepatocyte functions are not assessed.

Recent studies have been done to investigate the potential of umbilical cord blood and placental-derived cells [31], which have the obvious advantage of accessibility, as a source of hepatocyte precursors. Most studies transplant human cord blood cells directly into the livers of injured mice. They subsequently show the emergence of human derived hepatocytes which repopulated the injured livers [32-34]. Kakinuma et al showed that, in the presence of a variety of growth factors, up to 50% of the differentiated cord blood cells expressed albumin and these cells were able to proliferate *in vitro*. Although there is some promising data umbilical cord blood differentiation research is in its infancy and not well characterized.

1.6 Embryonic Stem Cells

An alternative cell source are embryonic stem (ES) cells. Pluripotent ES cells represent a promising renewable cell source to generate a variety of differentiated cell types including cardiomyocytes [35], neural precursors [36] and hepatocyte lineage cells [37], and may ultimately be incorporated into implantable engineered tissue constructs, or *ex vivo* cell based therapeutic devices[1, 2, 38, 39]. ES cells are continuously growing cell lines isolated from the inner cell mass of blastocysts [40]. Several studies use human embryonic stem cells in differentiation experiments [41]. Human ES cells, however, come with the

moral controversy of using embryos for research and are more difficult to propagate than murine ES cells. Murine ES cells provide a reasonable model of differentiation that can ultimately be applied to human cells. In addition, they have been shown to differentiate *in vitro* into cardiomyocyte and neuron lineage cells [35, 36]. In fact, it has been shown that hepatic differentiation can be induced through co-culture with embryonic cardiac mesoderm and may be required for hepatic differentiation to occur [42]. ES cells can be propagated *in vitro* using two different culture systems. They can be cultured on feeder layers of mouse embryonic fibroblast cells or in a media containing leukemia inhibitory factor (LIF). Both will maintain cell proliferation in the undifferentiated state. The differentiation of ES cells is induced by removing the ES cells from culture containing a feeder layer or LIF.

When LIF is removed and differentiation begins ES cells form aggregates in suspension and mimic *in vitro* embryogenesis. These aggregates, known as embryoid bodies (EB), have been studied extensively irrespective of the specific differentiated cellular target. EBs can be formed *in vitro* using a number of different techniques such as formation of hanging drops, liquid suspension culture and methylcellulose culture. Irrespective of the starting method for EB formation, by Day 12 of the differentiation process all EBs are similar in size and phenotypic expression [43]. EBs, upon differentiation, are comprised of all three germ layers and, after unguided differentiation, produce many different cell types. Many platforms, including EB mediated, have been studied to specifically direct the differentiation of embryonic stem cells toward a hepatocyte lineage, mimicking the fetal liver developmental pathways mentioned earlier. These platforms can be broadly grouped in terms of temporal regulation through cytokine addition or spatial regulation using various extracellular matrix configurations.

Although the majority of ES to hepatocyte differentiation platforms involve EB formation, there have been a select few which do not. For example, Maguire et al used an alginate poly-L-lysine microencapsulation platform to differentiate ES cells into hepatocyte lineage cells in the absence of EB formation [44, 45]. In these studies function was characterized by urea secretion as well as intracellular albumin expression and secretion. Interestingly, aggregation of cells in the capsules was found to be integral for the control of differentiation [45]. In addition, studies have been done to evaluate the effects of sodium butyrate on

hepatocyte differentiation from monolayer ES cell cultures. These studies assessed hepatocyte like differentiation via intracellular ALB, cytokeratin 18 (CK18) and glycogen staining. Urea secretion was also increased in these cultures [46]. An additional study, which exposed cells to sodium butyrate followed by a myriad of growth factors including HGF, OSM and DEX to further induce hepatocyte specificity, was also characterized via intracellular markers such as ALB and CK18 and upregulated hepatocyte lineage mRNA [47]. Teratani et al showed that ES monolayer cultures plated on thin layer gelatin, and subsequently collagen, while being treated with retinoic acid, FGF, HGF and OSM produced ALB expressing cells. The ES cell line used had been stably transfected with a pALB-EGFP gene which causes the cell to fluoresce when the ALB gene was being expressed. After the differentiation protocol ALB expressing cells were separated via fluorescence activated cell sorting (FACS) and subsequently implanted into mice with liver injury. This procedure increased survival times from 8 to 16 weeks [48]. The use of pALB-EGP transfected cells to isolate hepatocyte lineage cells from heterogeneous differentiated populations has been used in the EB mediated differentiation systems as well and will be discussed later in this section.

A significant majority of groups to first report differentiation of hepatocyte lineage cells from an ES source employed EB mediated differentiation and used a variety of developmental marker genes, such as the HNF family of transcription factors, transthyretine (TTR), α-fetoprotein (AFP), α1-antitrypsin (AAT), ALB, tyrosine aminotransferase (TAT), glucose-6-phosphatase (G6P), tryptophan oxygenase (TO), and glyceralaldehyde-3-phosphate dehydrogenase (GAPDH), assessed via RT-PCR and cDNA microarray analysis, to explore the extent of hepatocyte differentiation [37, 49-55]. In addition, immunophenotypic studies were conducted using flow cytometry and marker genes such as ALB, AFP and CK18 were used to visualize intracellular expression. The differentiation process which mediates the transition of ES cells, via EB formation, into hepatocytes has been well studied and is the focus of the first portion of the work described in Chapter 2 of this document.

In 2001, Hamazaki et al [37] showed that they could differentiate mouse ES cells, via EB formation, into hepatocytes *in vitro*. This is one of the first studies to have shown that, *in vitro*, with the supplementation of a thin layer gelatin and exogenous growth factors, EB mediated differentiation of mouse ES cells produced

a population of albumin-positive cells of hepatic lineage. Using a specific sequence of growth factors that mimic embryonic liver development and extra cellular matrix coatings, they showed, using RT-PCR, that the cells differentiated in the presence of growth factors expressed mRNAs such as AFP, tyrosine TAT, G6P and ALB. However, they did not demonstrate the functional potential (protein secreting or detoxifying) of these cells. A common theme running through all similar experiments is the addition of growth factors and plating the EB's on different tissue culture matrices to mimic *in vivo* developmental processes and induce hepatocyte differentiation.

Chinzei et al [49] also report the differentiation of mouse ES cells, via EB formation, into functional hepatocytes. Exogenous growth factors were added on specific days in the differentiation cycle and the EBs were plated on gelatin coated plates, similar to the protocol used by Hamazaki et al RT-PCR was used to evaluate mRNA expression of their differentiated ES cells. They reported the expression of ALB, AFP and TAT mRNA. Using western blot analysis they show that ALB was produced from Day 15 to Day 18 of the differentiation cycle. Interestingly, they reported that there was no increased expression of mRNA or protein in EBs cultured in the presence of growth factors when compared to spontaneously cultured EBs. They also evaluated urea synthesis and immunohistochemical staining for ALB to demonstrate the functionality of their differentiated cells. Synthesis of urea was first observed on Day 12 of the cycle and peaked at Day 18. The *in-situ* staining of EBs, 21 days into the differentiation process, showed that the cells producing albumin were in the stratiform cells rather than the monolayer cells. Miyashita et al [56], also stained EBs in situ and found that the cells expressing ALB where in the peripheral region of the EB. One study demonstrated the functionality of their differentiated ES cells via the uptake of indocyanine green, which is an organic anion that is used to evaluate liver function, because it is eliminated exclusively by hepatocytes [57]. These were the first studies to identify differentiated hepatocyte like cells with hepatocyte functional markers. However, detoxification and ALB secretion capabilities were not explored and many of the markers used to identify hepatocyte lineage cells in these studies are also found in the visceral endoderm portion of the embryo.

The visceral endoderm, which does not form the developing liver within a differentiating EB, has been shown to express markers similar to the developing liver such as ALB, AFP, and TRT as well as a variety of developmental genes such as HNF4 and acute phase genes such as matrix metalloproteinases. These similarities in gene expression profiles compound the difficulties in identifying and characterizing the level of maturity of hepatocytes cells derived from EB mediated differentiation. It is therefore necessary to differentiate between genes specific to fetal hepatocytes and those of the fetal visceral endoderm. Asahina et al. identified cytochrome p450 7a1 (CYP7a1) as a hepatocyte specific gene which is only expressed in hepatic tissue [6]. The study then went on to show that EB differentiation produces cells of both the visceral endoderm and hepatocyte lineages.

To address the issue of separating visceral from definitive endoderm, Gouon-Evans et al have shown that EBs cultured in the presence of activin A and serum free conditions efficiently produce definitive endoderm progenitor cells characterized by co-expression of endoderm specific Foxa2 and c-Kit. These cells are then specified to the hepatic lineage with BMP-4, in combination with FGF. This differentiation system induces 45-70% of the population to express ALB and AFP. In addition these cells secrete ALB and store glycogen. These cells were injected into livers and shown to evolve into mature hepatocytes [58]. Others have also attempted to isolate hepatocyte lineage cells and inject them into the injured livers of mice. pALB-GFP transfected ES cells were used to form EBs which underwent differentiation in the absence of growth factors. The ALB positive cells were then isolated via FACS. The sorted population was shown to express ALB, AFP and G6P. The population was then transplanted into diseased livers of mice and shown to have repopulated the liver by 82 days after transplantation [59]. A protocol employing a combination of the previously reported techniques was used to show reversal of mouse hepatic failure using implanted EB derived hepatocyte lineage cells. In this protocol, EBs were first co-cultured with immortalized human liver nonparenchymal cells in the presence of activin A and FGF to induce definitive endoderm lineage. The EBs were then exposed to DMSO, HGF and DEX. The ALB positive cells were then sorted via expression of a pALB-GFP using FACS. This sort yielded ~70% ALB positive cells. The sorted cells expressed liver specific genes such as CYP7a1, secreted ALB and metabolized ammonia, lidocaine and diazepam. Treatment of 90% hepatectomized mice with scaffolds incorporating these cells

improved liver function and prolonged survival [60-62]. Although these studies do not report of teratoma formation, others have reported formation of teratomas in mouse livers transplanted with mouse embryonic EB derived cells [63]. This issue may need to be further investigated with the use of implantable tissue engineered constructs and extracurporial BALs.

1.7 Unresolved Issues

The majority of the previously reported studies, using western blot analysis and RT-PCR, have shown gene expression profiles for a variety of genes most commonly associated with liver differentiation such as, ALB and AFP and have used ALB-GFP promoters to isolate hepatocyte lineage cells. Although most include growth factors, it has also been shown that hepatocyte differentiation can occur spontaneously, without stimulus from exogenous growth factors [41]. In fact, there is almost no consensus on which platform to use to differentiate hepatocyte lineage cells from ES cells. Techniques range from encapsulation to co-culture and no two platforms are alike. The cells produced by these different culture methods express similar genetic mRNA, are phenotypicaly similar and are similar to recent studies which have included albumin secretion, urea secretion and CYP7a1 expression, which has been shown to be hepatocyte specific. However, they have not shown detoxification mediated by specific CYPs, which is critical for their use in BAL treatment. Another overlooked issue is that most EB studies do not utilize controlled cultures, where more than one EB was placed into a single culture environment. They have not evaluated the differentiation which occurs when culturing a single EB versus a system with many EBs affecting each other in various spatio-temporal configurations. Because cell-cell contact has proven important in studies which indicate that proximity to cardiac like beating cells is an important stage in *in vivo* liver formation, evaluation of different culture environments on the differentiation of a single EB without the interference of other EBs and without constraints on growth such as interference from plate walls is an important factor that seems to have been overlooked.

In addition, the mechanisms behind spontaneous and directed EB mediated differentiation are not clear. Researchers have shown that both techniques produce albumin expressing cells but have not explored the mechanisms behind expression. Instead, most choose to implant or inject these cells directly into injured

livers despite the fact that the effect that the implanted cells may have on other organs and teratoma formation has not been well studied. Another point that has been overlooked is the long term propogation of differentiated cells while still maintaining differentiated function. Propagation is essential for scale-up and will play an important role in generating the large cell mass required from the small number of hepatocyte precursors isolated from the whole EB population.

1.8 Significance

The need for a well characterized, homogeneous, sustainable, ES derived hepatocyte like cell forms the basis for the research reported here. The experiments were designed to first decouple the effects of growth factors and extracellular matrices in EB mediated differentiation. Upon identification of the condition which most effectively induces the differentiation of hepatocyte lineage cells, this population was then propagated in secondary culture in order to generate a large and functional cell mass. Morphogens and secondary culture configurations, known to promote fetal hepatocyte maturation and adult hepatocyte functional maintenance, were used to augment and/or maintain these functions for extended periods of time.

CHAPTER 2: CONTROLLED EB MEDIATED DIFFERENTIATION TO EXPLORE THE EFFIENCY OF HEPATOCYTE LINEAGE COMMITMENT IN FOUR PARALLEL CULTURE CONFIGURATIONS: INSIGHTS FROM GENE EXPRESSION PROFILES

2.1 ABSTRACT

Pluripotent embryonic stem (ES) cells represent a promising renewable cell source for the generation of functional differentiated cells. Previous studies incorporating embryoid body (EB) mediated stem cell differentiation have, either spontaneously or following growth factor (GF) and extracellular matrix protein (ECM) supplementation, yielded populations of hepatocyte lineage cells expressing mature hepatocyte markers such as albumin (ALB). In an effort to promote ES cell commitment to the hepatocyte lineage, we have evaluated the effects of four culture conditions on albumin and gene expression in differentiating ES cells. Quantitative *in-situ* immunofluorescence and cDNA microarray analyses were used to describe not only lineage specificity but also to provide insights into the effects of disparate culture environments on the mechanisms of differentiation. The results of these studies suggest that spontaneous and collagen mediated differentiation induce cells with the highest levels of ALB expression but mature liver specific genes were only expressed in the spontaneous condition. Further analysis of gene expression profiles indicated that two distinct mechanisms may govern spontaneous and collagen mediated differentiation.

2.2 INTRODUCTION

Pluripotent embryonic stem cells (ES) represent a promising renewable cell source for generating a variety of differentiated cell types including cardiomyocytes [35], neural precursors [36] and hepatocyte lineage cells [37], and may ultimately be incorporated into implantable engineered tissue constructs, or *ex vivo* cell based therapeutic devices [1, 2, 38, 39]. *In vitro* aggregation of murine ES cells initiates the formation of embryoid bodies (EBs), which can facilitate spontaneous, unguided differentiation analogous to that seen in developing mouse embryos. During mouse embryogenesis, the primitive ectoderm gives rise to three distinct embryonic germ layers: definitive endoderm, mesoderm and ectoderm [64, 65]. The primitive endoderm gives rise to both the visceral and parietal endoderm and, while the definitive endoderm ultimately differentiates into mature hepatic tissue, the visceral endoderm is an extra-embryonic tissue, which expresses many genes also commonly expressed in differentiating liver cells [6].

Analyses characterizing the extent of EB differentiation have been limited to one or several lineage specific protein or gene expression patterns. For example, following extracellular matrix (ECM) and exogenous growth factor supplementation, several groups of investigators have demonstrated EB mediated differentiation of a population of albumin-positive cells [37, 49, 53, 66], or cells which express genes for ALB, AFP and TRT [50, 51, 54, 56, 57]. Spontaneous differentiation of human EBs into hepatocyte lineage cells, in the absence of both ECM and exogenous growth factor supplementation, has also been identified using gene microarray analysis of several known mature liver specific proteins [41, 67]. Despite the growing number of studies reporting ES/hepatocyte differentiation, isolation of pure populations of neither committed hepatocyte precursors nor fully differentiated hepatocytes have been reported. Furthermore, it is unclear whether both spontaneous and directed EB mediated differentiations are controlled by the same regulatory pathways or whether they yield identical hepatocyte lineage cells.

In the present study we will utilize controlled EB mediated differentiation to study ES cell differentiation into cells of the hepatocyte lineage. In order to decouple the effects of growth factors and collagen supplementation we will evaluate the effects of GFs alone, collagen alone, the presence of both and the absence of both on protein and gene expression levels. Recent studies have made advances using cDNA microarrays to identify kinetic expression of hepatocyte specific genes [55]. Here, in addition to a dynamic profile using *in-situ* staining of ALB protein expression and glycogen storage, we will use cDNA microarray analysis to assay expression of mature liver specific genes such as cytochrome P450 enzymes and cytokeratin 18 in the four differentiation conditions.

Identification of mature markers other than ALB and AFP is vital because these two most commonly used markers for the identification of mature hepatocytes are also expressed in the visceral endoderm of the differentiating embryo and may not be indicative of hepatocyte lineage cells. In addition to the identification of mature liver specific genes, an in depth analysis of the whole genetic expression profile of each condition will be done to clarify the basic differences in the mechanisms of differentiation. This data may be used with Pathway Assist software to construct regulatory pathways that may help to evaluate the gene expression data and give insights into regulatory pathways activated in different culture conditions.

The large amount of data generated by cDNA microarrays will help identify the condition with the greatest hepatocyte lineage commitment and reveal key differences in the mechanisms of ALB expression in the different conditions.

Upon the conclusion of chapter 2 we will have decoupled the effects of both growth factors and extracellular matrix supplementation on EB mediated differentiation of hepatocyte lineage cells. Identification of the culture condition with the highest levels of hepatocyte lineage commitment will enable us to identify the best condition to isolate a population of hepatocyte lineage cells for further analysis. In addition, we will gain further insight into the mechanisms regulating EB mediated differentiation in the different culture conditions. This may help engineer better differentiation systems in the future.

2.3 METHODS AND MATERIALS

2.3.1 ES Cell Culture

All cell cultures were incubated at 37 C° in a humidified 5% CO_2 incubator. The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated dishes (Biocoat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's modified Eagles medium (Gibco, Grand Island, NY) containing 15% knockout serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), 10 ug/ml gentamicin (Gibco), 1000 U/ml ESGRO (Chemicon, Temecula, CA), and 0.1mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Media was changed every two days. In order to induce differentiation, cells were suspended in Iscove's modified Dulbecco's medium containing 20 % fetal bovine serum (Gibco), 4mM L-glutamine (Gibco), 100U/ml penicillin, 100 U/ml streptomycin (Gibco), and 10 ug/ml gentamicin (Gibco). Embryoid bodies were formed and cultured for two days using the hanging drop method ($1x10^3$ ES cells per 30 ul drop). Hanging drops were transferred to suspension culture in 100 mm petri dishes and cultured for an additional 3 days. The EBs were then plated, one EB per well, in 6 well plates coated with, or without, collagen type I (40-60 µg/ml, Discovery Labware, Bedford, MA). When growth factors were supplemented, 100 ng/ml acidic fibroblast growth factor (aFGF) (Days 8-11) and 20 ng/ml hepatocyte growth factor (HGF) (Sigma, Saint Louis, MO) (Days 11-17) were added to the culture medium. Growth factors were selected to induce early hepatocyte commitment and not late

stage differentiation. The Hepa 1-6 cell line (ATCC, Manassas, VA) was maintained in Dulbecco's modified Eagles medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), and 4 mM L-glutamine (Gibco).

2.3.2 EB size

EB size was evaluated as a function of cell number/EB. Individual EBs were trypsinized and cells were counted. Twelve EBs were counted for each experimental day and the experiments were repeated three times. The same procedure was followed for all culture conditions.

2.3.3 In Situ Indirect Immunofluorescence

On Evaluation Days 11, 14, 17, EBs were washed in phosphate buffered saline (PBS) (Gibco) and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. The EBs were washed twice for 10 min in cold PBS and then twice for 10 min in cold saponine/PBS (SAP) membrane permeabilization buffer containing 1% bovine serum albumin (BSA) (Sigma-Aldrich), 0.5% saponine (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich). The EBs were subsequently incubated for 30 minutes at 4^{o}C in a SAP solution containing rabbit anti-mouse albumin antibody (150 ug/ml) (MP Biomedicals, Irvine, CA), or normal rabbit serum (NRS) (150 ug/ml) (MP Biomedicals) as an isotype control of the background intensity generated by non-specific binding, washed twice for 10 min in cold SAP buffer, and then treated for 30 minutes at 4^{o}C with the secondary antibody, FITC-conjugated donkey anti-rabbit, diluted 1:500 (Jackson Immuno Labs, Westgrove, PA). EBs were then washed once with cold SAP buffer and once with cold PBS. Fluorescent images were acquired with an Olympus IX70 microscope and Olympus digital camera using an excitation filter of 515 nm. Fluorescent albumin expressing cells were only observed in the outer EB region and not in the dense intermediate region or the central core. Image fluorescence was quantified with Olympus Microsuite. To generate intensity values for each of the samples, 10 cells located within this outer monolayer EB region were chosen at random from the phase contrast image. The average intensity value for 10 randomly chosen cells was measured in the NRS (control) case and this average background intensity was then subtracted from each value of the 10

randomly chosen cells in the anti-albumin (experimental) case. This was done in triplicate experiments yielding a total of thirty cells per condition.

Each data point represents the mean, and the error bars represent the standard error of the mean. Statistical significance was determined using the student t-test for unpaired data. Differences were considered significant when the probability was less then, or equal to, 0.05. Each experiment was repeated a minimum of three times, with a minimum of three replicates per experiment.

2.3.4 RNA Preparation for Microarray Analysis

RNA was prepared from ES and EB derived cells. Cells were homogenized with a tissue grinder in TRIzol (Invitrogen). Chloroform was added to the TRIzol homogenate to separate phases, then the aqueous phase was removed, mixed with an equal volume of 70% ethanol, and loaded onto an RNeasy column (Qiagen, Valencia, CA). The column was washed and RNA eluted according to manufacturer's recommendations. RNA was subjected to spectroscopic analysis of quantity and purity, with A_{260}/A_{280} ratios, at pH 8.0, between 1.9 and 2.1 for all samples. All RNA samples were subjected to capillary electrophoresis on an Agilent 2100 Bioanalyzer (Palo Alto, CA), with all samples demonstrating sharp 18S and 28S ribosomal RNA bands.

2.3.5 Microarray Design, Printing and Processing

The 21,997 probes on the custom microarrays contained a collection of oligonucleotides specific for mouse cDNA clusters purchased from Compugen/Sigma-Gnesis Oligos. The probes, 65–70 nt in length, were standardized for melting temperature and homology was minimized. Microarrays were printed at the Rutgers University Keck Center on poly-L-lysine-coated glass slides using an OmniGrid microarrayer (GeneMachines, San Carlos, CA) and quill-type printing pins. Poly-L-lysine slides were prepared and scanned at 532 nm and 635 nm in an Axon GenePix 4000B Scanner (Axon Instruments, Union City, CA) to evaluate surface quality. Slides were stored in a bench-top desiccator for at least 3 weeks prior to use. Oligonucleotides were resuspended to 40 μM in 3x SSC and rehydrated with shaking at room temperature. Printing was performed at 24°C with a relative humidity of ~50%. After printing, arrays were stored

overnight in a Parafilm-sealed plastic slide box in a desiccator at room temperature and postprocessed by standard procedures. Slides were stored at room temperature in a sealed plastic slide box in a desiccator and used for up to 3 months after printing.

2.3.6 Hybridization

Fluorescent probes were prepared using the Genisphere 3DNA dendrimer system (Genisphere, Hatfield, PA). Two micrograms of total cellular RNA was reverse-transcribed from a "capture-sequence"-containing oligo-d(T)18 primer using SuperScript II (Invitrogen). Following alkaline hydrolysis and neutralization, the cDNA target was hybridized with probes on the array at 58°C for 12 hours using a Ventana Discovery workstation (Ventana Medical Systems, Tuscon, AZ) and washed to high stringency (6). Dye- and capture sequence-specific fluorescent dendrimers (Genisphere) were then hybridized at 58°C for 2 hours. The arrays were washed after removal from the Discovery workstation and scanned on an Axon GenePix 4000B.

2.3.7 Microarray Data Analysis

Intensity values were obtained from the 16-bit tif. images generated from the scanner using TIGR Spotfinder (TIGR, Rockville, Maryland). A quality control check for spot size (using the Otsu algorithm) and intensity was applied at this point. Spots smaller than 2 pixels in diameter, larger than 25 pixels in diameter, or with fewer than 50% of the pixels being higher then 2 standard deviations of the background were flagged for later filtering. Local background values were also subtracted from spot intensity values using TIGR Spotfinder. Intensity values were then normalized per channel per chip using the Lowess function contained in TIGR Midas (TIGR, Rockville, Maryland). The normalized data set was passed through a series of two filters to obtain a list of annotated genes that demonstrated differential expression in intensity between the experimental and control case in each of the respective experimental scenarios. In filter 1, genes are discarded in each experimental condition if any replicate within either the cy3 or the cy5 data set did not pass the aforementioned TIGR Spotfinder quality control check. The genes that passed this criterion were subjected to a second filter where ANOVA was performed to test each gene independently for a statistical difference in expression between the experimental condition and its respective control. The

output of the analysis is the probability (*P* value) that a difference in expression can be observed by chance i.e. probability of getting a false positive. Although the occurrence of false positives can be controlled by choosing a higher significance level (0.01 or 0.05), it also concomitantly increases the false negatives. Therefore, in this study we have chosen to work with an ANOVA *P* value cutoff of 0.1. However, the large majority of genes reported here had *P* values below 0.05. To calculate the *P* value, we created an algorithm using the VBA package in Excel (Microsoft, Redmond, Washington) which utilizes a two sample, one-way ANOVA, analogous to a global two-tailed t-test. Three biological replicates were performed for each differentiation condition.

2.3.8 Gene Network Construction

Signaling pathways were modeled using Pathway Assist software (Ariadne Genomics, Rockville, MD). The PathwayAssist package utilizes a natural language algorithm (MedScan) to extract information on gene interactions from the ResNet database. In our analysis, we utilized the shortest path approach to link differentially expressed genes, with filters inclusive for protein nodes as well as regulatory controls.

2.4 RESULTS

Experiments were designed to establish a controlled EB mediated differentiation system to explore hepatocyte differentiation. Studies have shown that hepatocyte differentiation can occur in a variety of culture conditions including both guided, through growth factors and ECM supplementation, as well as spontaneous, non supplemented conditions [37, 41]. To decouple the effects of costly growth factors and extracellular matrix supplementation on hepatocyte lineage commitment, we evaluated four culture conditions in parallel.

2.4.1 Dynamic Studies of Hepatocyte Function Following ES Differentiation

Cultures were established to study the differentiation of individual EBs in the absence of outer wall mechanical constraint or interacting EB interference (**Figure 2.1**). Hepatocyte lineage commitment was initially assessed by examining the dynamics of albumin expression following LIF removal, within the four

culture environments: spontaneous, thin layer collagen supplementation (C), FGF and HGF supplementation (GF) and a combination of thin layer collagen and growth factor supplementation (C+GF). One EB was plated into the center of a well and evaluated on Days 11, 14 and 17 post plating. Cell number continued to increase throughout the 17 day culture period but the cells did not reach the outer well boundary (**Figure 2.2A**). Cell numbers, approximately $1.2x10^5$ cells per EB, were similar in all differentiation conditions. At the end of the culture period, the EB was characterized by a variety of cell morphologies contained outside the dense center core. Cells were rounded and densely packed within the intermediate region located adjacent to and surrounding the center core. The outer region was populated by elongated cells (**Figure 2.2B**). These regions generally did not become pronounced until Day 11 post-differentiation induction.

Experiments were designed to evaluate the differentiation of individual cells within the EB population and outside the EB central core by assessing *in situ* intracellular albumin expression. EB cultures were initiated as outlined above and albumin levels were qualitatively assessed on Day 17 post differentiation induction using indirect immunofluorescence with either primary anti-albumin antibody or an immunoglobulin control serum and subsequently fluorescently labeled secondary antibody. Images were captured using digital microscopy in order to determine the location of albumin expressing cells within the EB. As depicted in **Figure 2.3**, albumin expression was apparent within the EB outer region and not in the core or dense intermediate region, in both the spontaneous and C conditions. C+GF cultured cells expressed intermediate albumin levels. Albumin was not detected within either undifferentiated ES or GF cultured cell populations.

A dynamic profile of intracellular albumin expression was then established by quantifying the relative fluorescence intensity values in the digitized images (**Figure 2.4A**). Intracellular albumin was expressed on Day 11 in all differentiation conditions but expression was not maintained in the GF condition. In fact, albumin levels were negligible in GF cultured cells by Day 17 where only 10 % of the cells outside the EB core were albumin positive (**Figure 2.4B**). In contrast, both C and spontaneous cultures expressed the highest albumin levels in 80 % of the evaluated cells on Day 17, while C + GF cultured cells expressed

intermediate levels, with only 33 % albumin expressing cells outside the EB core. *In-situ* stains identifying glycogen storing cells at Day 17 have shown that glycogen is present in numerous cells in the EB population but unlike albumin expressing cells, glycogen expressing cells are located in the dense intermediate region of the EB in both the spontaneous and collagen conditions. (**Figure 2.5**)

2.4.2 Microarray Analysis

In order to better evaluate maturational commitment to the hepatocyte lineage within the four culture environments, cDNA microarrays were used to generate gene expression profiles 17 days post differentiation induction. The entire EB population was analyzed following EB incubation with trypsin and cell recovery. It is important to note that at later stages of differentiation, the inner central core was resistant to trypsin treatment and therefore these cells were not included in the analysis. Differential gene expression was measured using a 22k complete mouse cDNA microarray and gene expression within differentiated cells quantified relative to undifferentiated ES cells. Data analysis revealed that 3079 genes were differentially expressed in the spontaneous condition, 2079 genes were differentially expressed in the GF condition, 1137 genes were differentially expressed in the C condition and 2053 genes were differentially expressed in the C+GF condition relative to control ES cells (**Table 2.1**).

2.4.3 Hepatocyte Lineage Gene Expression

Hepatocyte lineage differentiation was assessed by examining differential expression of liver specific genes. Data analysis indicated that spontaneously cultured cells upregulated 7 cytochrome P450 enzymes expressed in mature liver cells and downregulated fetal liver cytochrome P450 3A13. In addition, these cells expressed cytokeratin 18, a hepatocyte specific cytoskeletal intermediate filament, cadherin 17, a liver and intestine cadherin, and transthyretin. Alcohol dehydrogenase 1 and aldehyde dehydrogenase 3A2 were upregulated in both the spontaneous and GF conditions (**Table 2.2**). Interestingly, of the 174 genes expressed in both the spontaneous and C conditions, which express albumin at almost equal levels on Day 17, 138 or ~80% of the genes upregulated in one condition were downregulated in the other (data not shown). In fact, although albumin was expressed in Day 17 C cultured cells, mature hepatocyte genes were

not. The disparity between the differentially expressed genes in these two conditions suggested that different differentiation mechanisms may result in similar albumin expression levels.

2.4.4 Regulation of Hepatocyte Lineage Commitment

In an effort to probe the mechanism of *in vitro* EB differentiation, we examined the expression of genes known to regulate liver development during normal *in vivo* embryogenesis, including growth factors and their receptors, ECM proteins, cell-matrix adhesion proteins and cell-cell adhesion proteins [68, 69]. FGF2, HGF and FGF receptor 1 were only expressed in the spontaneous condition, as shown in **Table 2.3**. FGF 2 receptor was upregulated in the spontaneous condition as well as in the GF condition. Exposure to collagen significantly decreased the number of differentially expressed growth factors and growth factor receptors.

Expression profiles of the fibronectin, laminin, and procollagen families of ECM proteins were also investigated. Fibronectin 1 was upregulated in both the spontaneous and GF conditions but the expression was significantly greater in the spontaneous condition. Laminin expression was up or down regulated in the spontaneous, GF and C+GF conditions but expression was unchanged in the C condition. Ten different members of the procollagen gene family were differentially expressed in the spontaneous condition but only two were upregulated. A variety of cell adhesion genes, notably integrins and cadherins, were also differentially expressed (**Table 2.3**). Thus, spontaneously differentiating cells, which expressed the most mature hepatocyte genes, also expressed the largest number of genes known to direct the differentiation of mature hepatocytes during embryogenesis [3, 68, 69].

2.4.5 Acute Phase Gene Expression

In order to gain further insight into the differentiated state of albumin producing EB cells, acute phase genes, known to be expressed in both regenerating liver and in primitive endodermally derived cells, were examined. Interleukin 1 alpha (IL1α) was upregulated in both C and C+GF conditions and interleukin 6 and tumor necrosis factor were downregulated in the spontaneous condition. In addition, cAMP responsive element (CREMP), activator protein 1 (AP-1) and STAT 1 genes were upregulated in the C+GF condition. Extracellular matrix remodeling genes, metalloproteinase 2 and 9, were also upregulated in the C+GF

condition. These results are summarized in **Table 2.4**. The data suggest that both C and C+GF cell populations, which express albumin and acute phase but not mature hepatocye genes, represent a maturational stage which is dramatically different from spontaneously differentiating cell populations.

2.4.6 Regulation of Albumin Expression

Our experimental results indicated that two conditions, spontaneous and C, induced high levels of albumin expression. However, while the spontaneous condition expressed hepatocyte specific genes, the C condition expressed genes commonly expressed during the acute phase response. In order to gain further insight into albumin regulation in these conditions, Pathway Assist software was used in conjunction with differentially expressed genes identified by our microarray analysis. This program generates regulatory pathways for input genes, upregulated in these conditions, from gene and protein interactions defined in current literature. As shown in **figure 2.6A**, albumin expression in the spontaneous condition is regulated by HGF and FGF. Interestingly, although epidermal growth factor (EGF) and insulin (INS) were not differentially expressed in our system, these genes were identified as key mediators in this pathway. In contrast, a distinct albumin regulatory pathway was identified in the C condition (**Figure 2.6B**). Albumin expression in the C condition was found to be potentially regulated by IL1 and IL2 and albumin in turn could regulate matrix metalloproteinase 9.

2.5 DISCUSSION

The development of implantable engineered liver tissue constructs and *ex vivo* hepatocyte based therapeutic devices are limited by an inadequate hepatocyte cell source. Pluripotent embryonic stem cells may solve this cell source limitation problem but their utility is contingent upon correct functional characterization and isolation. In the present study, we compared the effectiveness of guided and spontaneous differentiation from ES cell populations to generate a population of committed hepatocyte precursers. The present studies indicate that hepatocyte lineage commitment, characterized by a combination of hepatocyte specific gene expression and albumin production, was induced in the absence of both growth factors and ECM supplementation. In addition, while collagen supplemented cultures produced albumin, their gene profiles and associated regulatory pathways are distinct from those of spontaneously induced cultures.

While others have reported spontaneous differentiation of hepatocyte lineage cells [20, 21], the mechanism of differentiation induction has not been reported. *In vivo*, FGF is known to direct embryonic liver development. In fact, during *in vivo* mouse embryogenesis, induction of hepatic gene expression from the definitive endoderm is initiated following FGF signaling[3]. Cells committed to the hepatocyte lineage subsequently respond to HGF and other late stage growth factors to complete their functional differentiation [26, 70]. In addition, *in vitro* ES cells differentiated in the presence of cardiac mesoderm, an FGF producing tissue, induce differentiation of neighboring cells into hepatocytes [42, 69]. The results of our microarray analyses indicate that FGF2, HGF and FGF1 receptors were all upregulated in spontaneously differentiating EBs, but not in the other differentiation conditions tested. This suggests that an autoregulatory growth factor mechanism, similar to that of FGF stimulated embryonic liver development, controls spontaneous EB differentiation and likely parallels normal embryonic development. FGF10, FGF17 and FGF receptor 2 regulation was also observed in other culture conditions. The data also suggest that the addition of this growth factor cocktail induces the upregulation of FGF10 and that collagen supplementation suppresses the expression of FGF receptor 2.

Interestingly, when both aFGF and HGF were supplemented to the EB cultures, either in the presence or absence of collagen, albumin expression actually diminished and/or mature heptocyte genes were not expressed. In fact, in the absence of ECM supplementation, the addition of aFGF initially increased albumin expression on Day 11, but expression was not sustained following aFGF removal and subsequent HGF addition. This data suggests that optimum concentrations of growth factors and/or their receptors, may be auto-regulated in the spontaneous EB culture and that the growth factor cocktail added in these experiments voids the autoregulatory EB response. It is important to note that in our system, EBs cultured on collagen coated surfaces in the presence of these growth factors generated cells with significantly less albumin than either spontaneous or collagen mediated differentiation and did not express mature hepatocyte genes. However, in our studies we evaluated only early (aFGF) and mid stage (HGF) growth factors in order to evaluate commitment to the hepatocyte lineage. In contrast, others such as Hamazaki et al added late stage growth factors in addition to the ones used here, to induce end stage differentiation. Further

studies will evaluate the effect of late stage growth factors such as ITS and Oncostatin M (OSM) on either functional augmentation or terminal differentiation of cells isolated from either the spontaneous or supplemented cultures.

Many investigators have measured ALB, AFP and TRT expression in order to identify hepatocyte lineage cells. However, although these markers are widely used to asses function of mature hepatocytes isolated from adult livers, they may also be expressed in non-hepatic lineage embryonic cells [50, 51, 54, 56, 57]. In addition, other markers often used in evaluating hepatocyte differentiation, such as glucose-6-phosphatase, have also been found, using RT-PCR, in cells of a variety of other tissues including pancreatic islets [71]. In general, although RT-PCR is a powerful tool, only a limited number of genes are generally evaluated and many of these genes are expressed in a variety of embryonic and adult tissues. Because EB differentiation produces a very heterogeneous population, we have elected to evaluate gene expression within the entire mouse genome. While this approach may not be as quantitative as PCR, it provides a comparative platform of gene expression among parallel experimental cultures. The results of these analyses indicate that although high levels of albumin were expressed in both the spontaneous and C conditions, and intermediate albumin levels were expressed in C+GF condition, mature hepatocyte genes were only expressed in spontaneously differentiating cells. This suggests that albumin expression can be regulated by disparate mechanisms in these culture conditions and that distinct albumin expressing cell lineages may be differentiated in unique culture configurations.

In addition, EB cultures can yield not only hepatocyte lineage cells but may also induce albumin expressing visceral endodermal lineage cells of the primitive endoderm [10]. This is also suggested in our studies by the expression of many acute phase response genes in EB derived cells and is supported by the Pathway Assist analysis suggesting distinct albumin regulatory networks in the different culture conditions. While primitive endodermal cells may initially be generated in the spontaneous culture condition as well, these cells may be replaced by the differentiating hepatocyte lineage cells in this culture configuration. A dynamic gene array study will assist in elucidating both the differentiation profile and the pathways that may be activated during hepatocyte differentiation of ES cells.

Since neither the C+GF nor the C condition expressed a significant number of mature hepatocyte genes, we explored alternative mechanisms to explain the appearance of embryonic albumin producing cells. IL1α, in conjunction with collagen, has been shown to induce myofibroblastic activation, through matrix remodeling proteins such as matrix metalloproteinase 9 (MMP-9), during the liver acute phase response to injury [72]. In addition, expression of extracellular MMP's is developmentally controlled during the differentiation and spreading of the parietal endoderm [73]. In both C and C+GF conditions, gene expression of IL1α was significantly increased. While metalloproteinase was not upregulated in the C condition, the C+GF condition demonstrated not only increased MMP-9 gene expression but also a variety of other early acute phase genes listed in Table 4. These data suggest that the mechanism activated upon exposure to collagen may initiate upregulation of acute phase genes within primitive endodermal cells and ultimately an increased number of primitive endodermal cells within the EB. The effect was more pronounced in the C+GF condition, which also demonstrated decreased albumin expression and may therefore be regulated by the addition of early stage growth factors. Therefore, while collagen may initiate the acute phase response during EB differentiation, the effect may be more pronounced in the presence of growth factors.

Unlike the other culture conditions established in our studies, GF did not express albumin, acute phase genes or mature hepatocyte genes. While we can not definitively assign these cells to a specific embryonic lineage, they may represent a more primitive embryonic stage. It is also important to note that while identifying upregulated genes may assist in characterizing the cell lineage of differentiating cells, these data may not reflect actual protein synthetic levels. Future studies will assess the protein expression profiles of the genes identified in these studies. In addition, while we have identified hepatocyte lineage cells in spontaneously differentiating cultures, the incorporation of these cells into tissue constructs is contingent upon optimizing differentiated function and cell purification. The intrinsic heterogeneity of the differentiating EB makes characterization of a specific cell type difficult and further cell purification may also help yield a more accurate analysis. This is especially important since we have identified other lineage specific cells including nerve, lymphoid and muscle in our EB cultures (**Table 2.5**). In fact, even though

the dense inner region did not contain albumin expressing cells, it contains cells more reminiscent of mature hepatocyte morphology.

Nevertheless, our data indicate that spontaneous differentiation induces high ALB expression and liver specific gene upregulation. In addition, we have identified disparate pathways that may regulate albumin expression during EB differentiation. Experimental manipulation of these pathways may assist in engineering more efficient hepatocyte differentiation from ES cells.

CHAPTER 3: AUGMENTATION OF EB DIRECTED HEPATOCYTE-SPECIFIC DIFFERENTIATION VIA COALLAGEN SANDWICH AND SNAP

3.1ABSTRACT

The development of implantable engineered liver tissue constructs and *ex vivo* hepatocyte based therapeutic devices are limited by an inadequate hepatocyte cell source. In our previous studies, embryoid body (EB) mediated stem cell differentiation spontaneously yielded populations of hepatocyte lineage cells expressing mature hepatocyte markers such as albumin (ALB) and cytokeratin 18 (CK-18). In the present study, secondary culture configurations were used to study the effects of collagen sandwich culture and Oncostatin-M (OSM) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) supplementation of EB derived hepatocyte-lineage cell function. Quantitative immunofluorescence and secreted protein analyses were used to provide insights into the long term maintenance and augmentation of existing functions. The results of these studies suggest that SNAP, independent of the collagen supplementation, maintained the highest levels of ALB expression. However, mature liver specific CK-18 was only expressed in the presence of both gel sandwich culture supplemented with SNAP. In addition, albumin secretion and cytochrome P450 detoxification studies indicated that this condition was the best for the augmentation of hepatocyte-like function. Maintenance and augmentation of hepatocyte-like cells isolated from heterogeneous EB cell populations will be a critical step in generating large numbers of functional differentiated cells for therapeutic use.

3.2 INTRODUCTION

Acute liver failure affects hundreds of thousands of people per year around the globe and in many cases is resolved with an orthotopic liver transplant. Due to a shortage of donor organs, many patients die while waiting for a donor organ to become available. Extracorporeal liver assist devices (LAD) could help to bridge patients to transplant. However, this technology is limited by a lack of an adequate hepatocyte cell source [1, 2]. Pluripotent embryonic stem (ES) cells represent a promising renewable cell source to generate hepatocyte lineage cells, which have been incorporated into implantable engineered tissue constructs[61] and *ex vivo* cell based therapeutic devices such as LADs (Chul Cho). However, current

differentiation techniques have not yet generated the large and functionally sustainable cell masses which would be required to make such therapies clinically available.

ES differentiation into hepatocyte lineage cells, using a variety of differentiation platforms such as monolayer [46], encapsulation [44] and EB mediated [37, 59, 74], has been previously described by many investigators. Of these, EB mediated differentiation, which mimics *in vivo* embryogenesis, has been characterized most completely. For example, following exogenous growth factor supplementation and co-culture with nonparenchymal liver cell lines, investigators have demonstrated EB mediated differentiation yields of up to a 70% albumin-positive population, which expresses a variety of liver lineage genes and metabolizes lidocane and diazepam[62].

In addition, *in vitro* aggregation of murine ES cells initiates the formation of EBs, which has been shown to facilitate spontaneous differentiation in the absence of growth factor and extracellular matrix supplementation, resulting in liver lineage cells characterized by 80% albumin expression as well as mature hepatocyte genes such as cytochrome P450 detoxifying enzymes (CYP450) [75, 76]. However, despite the large number of studies reporting ES-hepatocyte lineage differentiation, there have been few reports of CYP450 related detoxification and drug metabolism which may be required for successful use of these cells for therapeutic treatment. Furthermore, maintenance of differentiated function and/or increased cell mass after the initial differentiation are critical steps for use of ES generated *in vitro* liver lineage cells in a LAD or drug discovery studies. However, these approaches have been largely ignored. Studies investigated the effects of Oncostatin-M (OSM) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) on fetal liver hepatocytes and have shown that their supplementation maintains long term structure and function as well as inducing further differentiation[77, 78].

In addition, it has been shown that, *in vivo*, terminal differentiation takes place after birth and is mediated through a combination of OSM, glucocorticoid, high-density culture and ECM [26, 70] Maintenance of function has been well studied in mature isolated hepatocytes and the conclusions of these studies may be applied to maintenance of differentiated stem cells. One notable study compared the function of adult

hepatocytes cultured on either collagen or Matrigel single gels and double gels in a sandwich configuration [13]. Collagen and Matrigel sandwich culture configurations sustained polygonal morphology, multicellular aggregate formation, and increased cell function. We see from here that ECM configuration is not only a major contributor to terminal differentiation but it also plays a significant role in maintaining in-vitro function.

In the present study, we have evaluated the effects of collagen sandwich culture and SNAP or OSM supplementation on maintenance of previously reported EB mediated spontaneously differentiated hepatocyte-lineage cell function. We have assessed expression and secretion of albumin within cells secondarily cultured from Day 17 EBs. In addition, using s-methylcholanthreene induction, we have assessed not only maintenance but also augmentation of function in the form of CYP450 mediated detoxification. These studies identified a secondary culture condition which maintained liver like function initially observed after 17 days of spontaneous EB mediated differentiation and furthermore promoted the detoxification functions of CYP450 enzymes.

3.3 METHODS AND MATERIALS

3.3.1 Cell Culture

All cell cultures were incubated in a humidified 37°C, 5% CO_2 environment. The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated flasks (Biocoat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's modified Eagles medium (Gibco, Grand Island, NY) containing 15 % knockout serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), 10 ug/ml gentamicin (Gibco), 1000 U/ml ESGRO™ (Chemicon, Temecula, CA), and 0.1mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). ESGRO™ contains leukemia inhibitory factor (LIF), which prevents embryonic stem cell differentiation. Every 2 days, media was aspirated and replaced with fresh media. Cultures were split and passaged every 6 days, following media aspiration and washing with 6ml of phosphate buffered solution (PBS) (Gibco). Cells were detached following incubation with 3ml of trypsin (Gibco) for three minutes, resulting in a single cell suspension,

and subsequently the addition of 12ml of Knockout DMEM. Cells were then replated in gelatin-coated T-75 flasks at a density of $1x10^6$ cells/ml.

In order to induce differentiation, cells were suspended in Iscove's modified Dulbecco's medium (Gibco) containing 20 % fetal bovine serum (Gibco), 4mM L-glutamine (Gibco), 100U/ml penicillin, 100 U/ml streptomycin (Gibco), and 10 ug/ml gentamicin (Gibco). Embryoid bodies were formed and cultured for 2 days using the hanging drop method ($1x10^3$ ES cells per 30ul drop). Hanging drops where transferred to suspension culture in 100 mm petri dishes and cultured for an additional 2 days. The EB's were then plated, one EB per well, in 6 well tissue culture polystyrene plates (BD-Biosciences) for an additional 14 days. For secondary culture, Day 17 EBs cells were detached following incubation with 0.5ml of trypsin (Gibco) for three minutes, resulting in a single cell suspension, and subsequently the addition of IMDM media. Cells were then re-plated in 6-well tissue culture polystyrene plates (BD-Biosciences) at an initial seeding density of $5x10^4$ Day 17 cells per well for further analysis. Culture medium was changed every forty-eight hours. When OSM and SNAP were supplemented, 10 ng/ml OSM and 250 μM SNAP were added to the culture medium. When collagen sandwich culture was used, rat tail type I collagen (BD-Biosciences) gels were prepared by distributing 350 μL of collagen gel solution (3 parts 1.33X DMEM, pH 7.4, and 1 part collagen solution at 4 mg/mL, chilled on ice and mixed immediately prior to use) evenly over one well of a six well plate (BD-Biosciences) and incubated at 37°C for at least one hour before use. $5x10^5$cells were seeded in 2 mL of IMDM media and an additional 350 μL of collagen gel solution was distributed over the cells after 1 day of culture. One hour of incubation at 37°C was allowed for gelation and attachment of the second gel layer before the medium was replaced. Culture medium was changed every forty eight hours.

The Hepa 1-6 cell line (ATCC, Manassas, VA) was maintained in Dulbecco's modified Eagles medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), and 4 mM L-glutamine (Gibco). Hepa 1-6 cells were grown on tissue culture treated T-75 flasks (Falcon, BD Biosciences, San Jose, CA). Hepa 1-6 cells were used as positive controls for each of the following assays.

On Evaluation Days 4, 6, 8 and 10 in secondary culture, cells were re-plated into 12 well plates. Media samples were collected after 24 hours of culture at 37°C and in 5% CO_2. The cells were then washed in PBS (Gibco) and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. Cells in collagen sandwich culture were dissociated with 0.5 mL of 0.1% collagenase (Sigma-Aldrich) in PBS for 30 minutes at 37°C before re-plating into 12 well plates.

3.3.2 In Situ Indirect Immunofluorescent Cytokeratin-18 and Intracellular Albumin Analysis

After 24 hours in culture and fixing with 4% paraformaldehyde, the cells were then washed for 10 min in cold PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 minutes at room temperature. The cells were washed twice for 10 min in cold PBS and then twice for 10 min in cold saponine/PBS (SAP) membrane permeabilization buffer containing 1% bovine serum albumin (BSA) (Sigma-Aldrich), 0.5% saponine (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich). To detect intracellular albumin, the cells were subsequently incubated for 30 minutes at 4^{o}C in a SAP solution containing rabbit anti-mouse albumin antibody (150 ug/ml) (MP Biomedicals, Irvine, CA), or normal rabbit serum (150 ug/ml) (MP Biomedicals) as an isotype control, washed twice for 10 min in cold SAP buffer, and then treated for 30 minutes at 4^{o}C with the secondary antibody, FITC-conjugated donkey anti-rabbit, diluted 1:500 (Jackson Immuno Labs, Westgrove, PA). To detect cytokeratin 18, which is produced in mature hepatocytes and a few other mature cell types, cells we incubated for 30 minutes at 4^{o}C in a SAP solution containing rabbit anti-mouse cytokeratin 18 antibody (IgG1) (1:50 dilution) (Santa Cruz Biotechnology) or the IgG1 fraction of normal rabbit serum (1:100 dilution) (Santa Cruz Biotechnology) as an isotype control, and then treated for 30 minutes at 4^{o}C with the secondary antibody, FITC-conjugated goat anti-rabbit, diluted 1:200 (Jackson Immuno Labs, Westgrove, PA). For both stains, cells were then washed once with cold SAP buffer and once with cold PBS. Fluorescent images were acquired using a computer-interfaced inverted Olympus IX70 microscope. Specimens were excited using a 515nm filter. Fluorescent intensity values were determined for each cell using Olympus Microsuite. Experimental intensity values for each cell were calculated after subtracting the average intensity of the isotype control.

3.3.3 Glycogen Staining

Following 24 hours in culture and fixing with 4% paraformaldehyde, cells were exposed to 0.25 ml of Periodic Acid Solution (Bittner, Meltzer et al.) (Sigma Aldrich) per well for 5 minutes at room temperature. Glycols are oxidized to aldehydes in this process, which is not entirely specific to hepatocytes. After washing cells with PBS to remove the PAS, 1ml of Schiff's reagent was added per well and cells were exposed for 15 minutes at room temperature. Schiff's reagent, a mixture of pararosaniline and sodium metabisulfite, reacts to release a pararosaniline product that stains the glycol-containing cellular elements. A third PBS wash to remove the reagent was followed by image acquisition with an Olympus IX70 microscope and Olympus digital camera.

3.3.4 Sandwich ELISA for Detection of Albumin Secretion

In order to detect secreted albumin within the media supernatants obtained on each of the analysis days, we used a commercially available mouse albumin ELISA kit (Bethyl Laboratories, #E90-134). A standard curve was generated by creating serial dilutions of an albumin standard from 7.8 to 10,000 ng/mL. Absorbance readings were obtained using a Biorad (Hercules, CA) Model 680 plate reader with a 450 nm emission filter. Albumin values were normalized to the cell number recorded on the day of media sample collection.

3.3.5 Urea Secretion

Media samples were collected on all analysis days. Urea synthesis was assayed using a commercially available kit (StanBio, Boerne, TX). A standard curve was generated by creating serial dilutions of a urea standard from 0 to 300 mg/mL. Absorbance readings were obtained using a Biorad (Hercules, CA) Model 680 plate reader with a 585 nm emission filter. Urea values were normalized to the cell number recorded on the day of media sample collection.

3.3.6 Measurement of Cytochrome P450 Activity

On Evaluation Days 4, 6, 8 and 10 in secondary culture, cells were re-plated into 12-well plates. 3-methylcholanthrene was used at a concentration of 2 μM (Sigma–Aldrich) for 48 hours prior to the addition

of resorufin as an inducer of cytochrome P450 activities. Cytochrome P450-dependent resorufin *o*-dealkylase activity (BROD, PROD, EROD, and MROD) was measured using resorufin substrates namely pentoxy-, benzyloxy-,ethoxy-, and methoxyresorufin from a Resorufin Sampler Kit (Invitorgen, Carlsbad, CA). The incubation mixture contained resorufin substrates (pentoxy-, ethoxy-,or methoxyresorufin, final concentration 5 *m*M) and dicumarol (80 *m*M) in phenol red free Earle's Balanced salt Solution (EBSS) (Gibco). The prepared solutions were preheated to 37°C, prior to incubation with cells. The 12 well plates were washed with 2 mL of EBSS (37°C) and further incubated with 2 mL of EBSS at 37°C for 5–7 min, to remove the residual medium. Following removal of EBSS, the incubation mixture was added (2 mL per well), and the dishes were incubated at 37°C in a 5% CO2 incubator. At various time points (5, 10, 15, 20, 25 min) following incubation, 100 μL of the mixture was transferred into a 96-well plate. The fluorescence of the plate was measured using a fluorescence plate reader (DTX880, Beckman Coltour, Fullerton, CA, ext. 530 nm and emis. 590 nm) at the end of 25 min incubation. A standard curve of resorufin fluorescence was constructed using concentrations ranging from 1 to 1,000 nmol in EBSS. A linear curve was obtained with an r^2 of 0.99. The constructed standard curve was used to convert the fluorescence values obtained from the plate reader to nanomoles of resorufin. Rate of formation of resorufin, as calculated from the early linear increase in the fluorescence curve, was defined as cytochrome P450 activity and expressed as nmol/min.

3.3.7 Statistical Analysis of Functional Assays

Each data point represents the mean of three experiments (each with three biological replicates), and the error bars represent the standard deviation of the mean. Statistical significance was determined using the student t-test for unpaired data. Differences were considered significant when the probability was less then or equal to 0.05.

3.4 RESULTS

3.4.1 Dynamic Studies of Secondarily Cultured EB Derived Hepatocyte Lineage Cells

Cultures were established to study the maintenance and augmentation of hepatocyte-like function previously observed after 17 days of spontaneous EB mediated differentiation. Hepatocyte lineage maintenance was initially assessed by examining the dynamics of cell growth following removal of cells from their primary EB culture and re-plating onto tissue culture polystyrene. $5x10^4$ cells from Day 17 EB cultures were re-plated into one well of a six-well plate and evaluated on days 4, 6, 8 and 10 days post re-plating. Cell number increased rapidly and confluence was reached at Day 6. Therefore, cells were re-plated into tertiary culture at $5x10^4$ cells per well and continued to proliferate for the next four days. (**Figure 3.1A**)

Next, experiments were designed to evaluate the maintenance of function seen in EB generated hepatocyte lineage cells by assessing *in situ* intracellular ALB and CK18 expression. Secondary and tertiary cultures were initiated as outlined above and ALB and CK18 expression were qualitatively assessed 4, 6, 8 and 10 days post re-plating using indirect immunofluorescence with either primary anti-ALB/CK18 antibody or an immunoglobulin control serum and subsequently fluorescently labeled secondary antibody. Images were captured using digital microscopy in order to determine the percent of ALB and CK18 expressing cells within the cultures**.** Day 17 EB generated cells were 80% albumin positive (**Figure 3.1B**) and 60% CK18 positive (**Figure 3.1C**). As depicted in Figure 1B, ALB expression was maintained for 6 days at ~40% in secondary culture, however, expression was not maintained past 6 days in tertiary culture. CK18 expression was maintained at minimal expression levels for 4 days in secondary culture but was absent on subsequent days. (**Figure 3.1C**) In secondary polystyrene culture, EB derived cells proliferated rapidly but could not sustain albumin expression. Therefore, we explored the addition of soluble factors on proliferation and maintenance of function.

3.4.2 OSM and SNAP Supplementation

In order to investigate the effect of soluble factors previously shown to affect hepatic function, re-plated cells were supplemented with either OSM or SNAP. Cell numbers in the OSM supplemented condition were similar to that of the un-supplemented cultures and increased dramatically in the OSM supplemented cultures. However, cells exposed to SNAP were generally characterized by slower growth rates. Due to the rapid growth seen in the un-supplemented and OSM cultures, at Day 6, cells were re-plated into tertiary culture at $5x10^4$ cells per well and continued to proliferate for the next four days. (**Figure 3.2A**) ALB expression was maintained in the OSM supplemented cultures for up to eight days in secondary culture. The cells supplemented with SNAP also maintained some ALB expression for up to eight days in tertiary culture but at a lower level. There was no significant expression following ten days in secondary culture in any condition. (**Figure 3.2B**) Urea secretion was greatest at Day 8 in the OSM supplemented condition however some secretion was detected at all experimental time points. Day 17 EB hepatocyte like cells exhibited a urea secretion rate of 50 $\mu g/10^6$ cells/day. (**Figure 3.2C**) A summary of the hepatocyte like functions tested is summarized in **Table 3.1**. CK18 expression, as well as other hepatocyte functions such as albumin secretion, glycogen storage and CYP450 mediated detoxification, was not detected at any level in the OSM or SNAP supplemented cultures. Although addition of soluble factors maintained albumin secretion for up to eight days in secondary culture, certain hepatocyte functions were not maintained at any significant level and others were totally absent.

3.4.3 Collagen Sandwich Culture

In order to determine whether we could further augment and/or maintain the function of the hepatocyte like cells isolated from Day 17 EB culture, collagen sandwich culture, a system which has been well studied for maintenance of mature hepatocyte function,[79] was utilized alone (GEL) and in conjunction with OSM (GOSM) and SNAP (GSNAP) supplementation. Cells cultured in a sandwich configuration were characterized by a slower rate of proliferation as compared to polystyrene culture. Cells in the GEL and GOSM conditions reached maximum growth at Day 6 and cells in GSNAP by Day 8. Because of low proliferation rates in sandwich culture, the cells did not reach absolute confluence and no tertiary culture was employed. (**Figure 3.3A**) As depicted in **Figure 3.3B**, albumin expression was detected in all

conditions for 6 days in secondary culture. However, expression was maintained at 80%, 10 days post re-plating, only in the GSNAP and GOSM conditions. A similar effect was evident in the expression of CK18 where some expression was detected in the non supplemented, non GEL (NS) condition for 4 days but it was expressed only in the GSNAP and GOSM conditions after 10 days. However, the ~45% expression in the GSNAP condition was significantly higher than the ~20% seen in the GOSM condition. (**Figure 3.3C**) The GEL, GOSM and GSNAP conditions stored glycogen 10 days into secondary culture. The NS conditions did not significantly stain for glycogen. (Data not shown)

Urea and albumin secretion, vital liver functions, were used to asses mature hepatocyte specific differentiated function. A dynamic profile of ALB secretion was established using qualitative ELISA analysis. Although at four days in secondary culture there was an initial induction of ALB secretion in both the GOSM and GSNAP conditions, rates were significantly higher in the GSNAP condition on subsequent days compared to all other conditions. In addition, secretion was maintained at 60 $\eta g/10^6$ cells/day after 10 days in secondary culture (**Figure 3.4A**). Day 17 EB derived cells did not secrete albumin (data not shown) and had a urea secretion rate of 50 $\mu g/10^6$cells/day. In secondary culture all conditions maintained some urea secretion. However, the 25$\mu g/10^6$ cells/day observed in the GSNAP condition was significantly higher than any other condition at Day 10. (**Figure 3.4B**)

At the end of the culture period, cells cultured in all conditions were characterized by a variety of cell morphologies. Cells were assembled in random densely packed groupings in all double gel conditions and exhibited tightly packed morphologies. However, in the GSNAP condition, there was a second morphology which was characterized by greater than 95% of cells in groups of round or square cells in a non confluent, loosely connected environment. (**Figure 3.5**)

3.4.4 Cytochrome P450 Detoxification

Cytochrome P450 enzymes play a key role in detoxifying xenobiotics and were used in these studies to assess hepatocyte function. The present studies monitored the expression and stabilization of benzyloxyresorufin *o*-dealkylase (BROD) and methoxyresorufin *o*-dealkylase following induction with 3-

methylcholanthrene for 48 hrs, in D17 EB derived cells as and for 10 days in secondary GSNAP culture. BROD and MROD activity can be determined from the enzymatic conversion of resorufin. This activity detected via increasing concentration of resorufin was only apparent after 10 days in secondary GSNAP culture (**Figure 3.6A**). The rate of production was similar to that of the Hepa 1-6 control. (**Figure 3.6B**)

3.5 Discussion

The development of implantable engineered liver tissue constructs and *ex vivo* hepatocyte based therapeutic devices are limited by an inadequate hepatocyte cell source. Differentiated pluripotent embryonic stem cells have been used to alleviate the cell source limitation problem but their utility is contingent upon generating the large number of cells and sustaining function for extended periods of time. In the present study we used previously identified hepatocyte lineage cells [75] to evaluate the effects of OSM, SNAP and collagen sandwich culture on maintenance and augmentation of differentiated function already observed after EB mediated differentiation. The present studies indicate that maintenance of function, characterized by intracellular ALB and CK18 expression as well as urea secretion, was maintained in the presence of SNAP and OSM when used in conjunction with collagen sandwich culture. In addition, while albumin secretion and CYP450 detoxification were not seen in the starting population, they were induced after 10 days in the GSNAP cultures.

While others have reported long term maintenance of hepatocyte-like function from ES sources differentiated *in vivo* [80] there have been no reports of maintaining EB derived hepatocyte like function after the primary differentiation is complete. In fact, most studies do not explore function past the initial differentiation protocol. A significant problem associated with *ex vivo* adult hepatocyte culture is the rapid loss of differentiated function and morphology.[81, 82] In our studies we saw a similar effect in that intracellular ALB and CK18 expression was significantly reduced when cultured under standard tissue culture conditions and passed several times into secondary and tertiary culture. In order to better study adult hepatocyte function, investigators have shown that when cultured in collagen sandwiches, hepatocytes maintain albumin secretion and cell morphology for up to 42 days [83]. In the present studies there was a marked difference in cellular morphologies observed in the collagen sandwich culture; however, collagen

sandwich culture alone could not maintain ALB expression past the 6 days in secondary culture and had limited CK18 expression.

Interestingly, when SNAP or OSM was added to the sandwich culture results were significantly altered. It is well known that nitric oxide triggers mitochondrial biogenesis in a variety of mammalian cells [84, 85]. In addition, NO and NOS isoforms have been shown to induce differentiation of nerve cells, tumor cells and cardiomyocytes [86-88]. The effect of SNAP, a nitric oxide donor, has been investigated in liver cell systems [89-92]. In this regard, we have utilized SNAP, a key regulatory molecule, to increase hepatic differentiated function. SNAP has been shown to upregulate mitochondrial and differentiated function in hepatocyte-like cells derived from embryonic stem cells (Sharma et al) and OSM has been identified as a key morphogen in the transition from fetal to adult hepatocytes [93] as well as the adult liver's regeneration in response to injury. [94]

Many investigators have measured ALB expression both using immunocytochemistry and RT-PCR in order to help identify hepatocyte lineage cells in a variety of differentiation protocols. [50, 56, 95] Recently, ES cells transfected with green fluorescent protein (GFP) reporter gene regulated by ALB enhancer/promoter have been used to identify and isolate hepatocyte lineage cells during differentiation [48, 51, 59, 61]. In these studies there have been reports of up to 70% albumin positive cells. However, as others have previously shown, because ALB may also be expressed in the visceral endoderm as well as fetal tissue, it alone can not be used to confidently identify hepatocyte lineage cells.[6] The combination of sandwich culture with SNAP supplementation not only maintained albumin expression at all time points but also resulted in over 80% ALB positive cells, indicating a relatively homogeneous population about four weeks after differentiation induction. In addition to ALB expression, CK18 expression was maintained at 45% up to 10 days in secondary culture. Although this is lower than the 60% positive population seen immediately following EB differentiation, the GSNAP culture condition was the only CK18 expressing condition. A similar trend was seen with urea secretion where, after 10 days in secondary culture, urea secretion in the GSNAP condition was not as high as in the Day 17 EB culture but was significantly greater than in any other culture condition.

In addition to maintaining ALB, CK18 and urea secretion, the GSNAP condition also induced albumin secretion not seen at any significant level in Day 17 EB culture. Albumin secretion from ES derived hepatocyte lineage cells has been reported previously [45, 58, 61, 76, 80] and in the current studies is first detected at Day four at 120 ηg/10^6 cells/ day and decreases to about 60 ηg/10^6 cells/ day. Although this is significantly lower than the levels of secretion seen in the Hepa1-6 control, it is significantly higher than any other experimental condition evaluated here and similar to previously reported ES derived hepatocyte-like secretion level.

In addition to secreted function, detoxification was also detected via CYP450 metabolism in the GSNAP condition. Xenobiotic metabolism has been well characterized in primary hepatocyte systems [96, 97] and, although there have been reports of induction of CYP450 mRNA in ES derived hepatocyte-like cells, there have been few reports of detoxification, a function which would be critical for use of these cells in a LAD. [6, 61, 76] Here we used 3-MC for 48 hours to induce CYP450 activities and observed that both BROD and MROD detoxification was observed at a level similar to the Hepa1-6 mouse hepatocyte carcinoma cell line. This is the first report of CYP450 mediated detoxification from ES derived hepatocyte like cells.

Interestingly, in the present analyses, most reported functions were seen after 10 days in secondary culture in the GSNAP condition. This allows us to maintain and augment function of spontaneously EB mediated hepatocyte like cells. In addition to maintenance, we were able to show an increase in cell number from $5x10^4$ Day 17 cells to $1x10^6$ cells 10 days into secondary culture while still maintaining 80% ALB expression which may represent an amplified hepatocyte-like population. The fact that Day 17 EB cells can proliferate in the GSNAP condition, while maintaining their hepatocyte-like characteristics, brings an added value to generating the large mass of cells required for use in a LAD. Future studies to characterize tertiary culture of GSNAP cells could uncover methods to further increase cell mass while sustaining function. Nevertheless, our data indicate a combination of maintenance and augmentation of hepatocyte specific functions in conjunction with an increase in cell mass in the GSNAP condition for up to four weeks post differentiation induction.

CHAPTER 4: FUTURE DIRECTIONS

4.1 SYNOPSIS

Stem cells have been shown to differentiate into cells of organs and tissues which arise from all three developmental germ layers. Their potential in the therapeutic arena, although immature, has had some initial success in the treatment of autoimmune diseases and anti inflammatory diseases. Embryonic stem cells in particular have been widely studied for their potential to differentiate into a renewable hepatic cell source. In this body of work we have demonstrated that the EB body platform can be used to differentiate cells of the hepatocyte lineage in the absence of growth factor and extracellular matrix supplementation. In addition, we have shown that cellular function observed after EB differentiation can be maintained and augmented in secondary collagen sandwich culture supplemented with SNAP. While the results we have obtained using the combination of primary EB and secondary collagen sandwich systems are very promising, a large body of work will be needed to translate these findings from the level of research and development to that of application within clinical and technological devices such as BALs, and *in vitro* drug screening systems.

4.2 FUTURE WORK

4.2.1 Field Consensus

One of the major issues which has not been well addressed is the wide spectra of differentiation platforms used to obtain hepatocyte like cells from ES cell sources. Of all the techniques which have been explored by other researchers and described here, there are no two platforms which are alike. Because of these disparities, very few platforms are well studied and instead of refining already existing platforms, we are creating new ones and starting over each time. Both the platform and the tools used to evaluate differentiated function should be narrowed and a consensus should be reached. In this respect, the culture systems identified in this thesis can be further probed to better understand the mechanisms of differentiation such as, why does the spontaneous EB differentiation yield cells with the most hepatocyte-like characteristics. Although this question has been addressed in the previous sections, it can be further probed and may lead to some consensus in the field.

More work can also be done to standardize EB formation. Formation is generally consistent; however, survival and proper differentiation of EBs are dependant on the starting population of ES cells. In this respect, it may be beneficial to evaluate a number of ES lines on EB formation and end product differentiation. Could results be kept consistent while varying the starting cell line? In addition, we might want to evaluate the effect of undifferentiated ES cell culture. As described previously, ES cells can be kept undifferentiated using culture with LIF supplemented media or through growth on irradiated feeder layers. It would be interesting to identify changes in the differentiated population, if they exist, derived from cells cultured using these techniques.

4.2.2 Scale-up and Maintenance of EB Derived Hepatocyte–Like Cells

One of the issues with using ES derived hepatocyte-like cells in therapeutic devices, as well as in drug toxicity studies, is the large cell mass needed. To sustain a murine BAL we would need to generate ~10^7 cells per device and ~10^{10} for a human device. Here we have shown that when we start with $1x10^3$ cells per EB, after 17 days of differentiation we have ~ $1.2x10^5$ cells. Taking these Day 17 cells and then placing them into secondary collagen sandwich culture for ten days, we obtain ~$2x10^6$ cells from one EB. Thus, generating a cell mass of 10^7 cells for use in a murine BAL would require the use of five EBs and is very plausible. In addition to generating the cell mass, the cells also need to be maintained for extended periods of time. Here we have shown maintenance for up to ten days in secondary culture. In the future, extending the effects of maintenance in secondary culture can provide researchers with a more sustainable and readily available cell source.

4.2.3 Using EB Differentiation to Derive Alternative Adult Cell Types

In addition to utilizing EB mediated differentiation as a tool to derive mature hepatocytes, studies have evaluated the capacity of the system for neuronal differentiation. As we have shown here, microarray data from Day 17 spontaneously cultured cells have shown that genes from a variety of cell lineages, such as neuronal and cardiac, were upregulated. Studies have also shown that cardiac mesoderm initiates the differentiation of the liver bud *in vivo* and that the two tissues are closely connected. These data indicate

that with the right culture configurations and manipulations, the spontaneously differentiating EB can yield cells of other lineages. These cells can then be isolated and used in therapeutic devices or transplants.

4.2.4 Alternative Stem Cell Sources

Throughout this project all studies have been conducted using a murine embryonic stem cell source. In order to truly be effective in an extracorporeal bioartificial liver, the source will have to have ceratin characteristics: 1) of human origin; and 2) does not pose the ethical issues that human embryonic stem cells do. As a new starting material, we are currently working with human umbilical cord blood cells, which can now be readily obtained for research purposes, as they are closer to our final product concept. Similar techniques to the ones described here could be applied to the study of these cells including EB mediated differentiation. I believe that because this platform most closely mimics *in vivo* differentiation it is an ideal technique for differentiating ES cells *in vitro*, independent of their original source. In addition, when a method for expansion of small populations becomes available, we could explore the use of hepatoblasts as well as oval cells, which would alleviate both ethical and immune response issues.

4.3 CONCLUSIONS

In summary, we have demonstrated that EB differentiation provides a system to control embryonic stem cell differentiation into hepatocytes. We have shown that, of the various EB mediated differentiation techniques, spontaneous differentiation produces the most hepatocyte-like cells. In addition, we have shown that function observed after 17 days of EB mediated differentiation can be maintained for up to ten days in secondary culture. Although there is much work to be done before ES cells will be used in human therapies, the platform described in this thesis can be used to test these cells in murine BALs, tissue engineered implantable constructs or drug discovery studies.

BIBLIOGRAPHY

1. Tilles, A.W., et al., *Bioengineering of liver assist devices.* J Hepatobiliary Pancreat Surg, 2002. **9**(6): p. 686-96.
2. Tilles, A.W., et al., *Critical issues in bioartificial liver development.* Technol Health Care, 2002. **10**(3-4): p. 177-86.
3. Jung, J., et al., *Initiation of mammalian liver development from endoderm by fibroblast growth factors.* Science, 1999. **284**(5422): p. 1998-2003.
4. Fukuda-Taira, S., *Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm.* J Embryol Exp Morphol, 1981. **63**: p. 111-25.
5. Rossi, J.M., et al., *Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm.* Genes Dev, 2001. **15**(15): p. 1998-2009.
6. Asahina, K., et al., *Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells.* Genes Cells, 2004. **9**(12): p. 1297-308.
7. Yasunaga, M., et al., *Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells.* Nat Biotechnol, 2005. **23**(12): p. 1542-50.
8. Okaya, A., et al., *Oncostatin M inhibits proliferation of rat oval cells, OC15-5, inducing differentiation into hepatocytes.* Am J Pathol, 2005. **166**(3): p. 709-19.
9. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz,, *The Liver: Biology and Pathobiology.* 1988, New York: Raven Press.
10. Hay, P.D., A.R. Veitch, and J.D. Gaylor, *Oxygen transfer in a convection-enhanced hollow fiber bioartificial liver.* Artif Organs, 2001. **25**(2): p. 119-30.
11. Hay, P.D., et al., *Oxygen transfer in a diffusion-limited hollow fiber bioartificial liver.* Artif Organs, 2000. **24**(4): p. 278-88.
12. Park, J., et al., *Microfabricated grooved substrates as platforms for bioartificial liver reactors.* Biotechnol Bioeng, 2005. **90**(5): p. 632-44.
13. Moghe, P.V., et al., *Culture matrix configuration and composition in the maintenance of hepatocyte polarity and function.* Biomaterials, 1996. **17**(3): p. 373-85.
14. Semler, E.J. and P.V. Moghe, *Engineering hepatocyte functional fate through growth factor dynamics: the role of cell morphologic priming.* Biotechnol Bioeng, 2001. **75**(5): p. 510-20.
15. Bhatia, S.N., et al., *Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions.* Biotechnol Prog, 1998. **14**(3): p. 378-87.
16. Hansen, L.K., et al., *Enhanced Morphology and Function in Hepatocyte Spheroids: A Model of Tissue Self-Assembly.* Tissue Eng, 1998. **4**(1): p. 65-74.
17. Parsons-Wingerter, P.A. and W.M. Saltzman, *Growth versus function in the three-dimensional culture of single and aggregated hepatocytes within collagen gels.* Biotechnol Prog, 1993. **9**(6): p. 600-7.
18. Theise, N.D., et al., *The canals of Hering and hepatic stem cells in humans.* Hepatology, 1999. **30**(6): p. 1425-33.
19. Evarts, R.P., et al., *A precursor-product relationship exists between oval cells and hepatocytes in rat liver.* Carcinogenesis, 1987. **8**(11): p. 1737-40.
20. Evarts, R.P., et al., *In vivo differentiation of rat liver oval cells into hepatocytes.* Cancer Res, 1989. **49**(6): p. 1541-7.
21. Nagy, P., et al., *In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene.* Hepatology, 1996. **23**(1): p. 71-9.
22. Shafritz, D.A. and M.D. Dabeva, *Liver stem cells and model systems for liver repopulation.* J Hepatol, 2002. **36**(4): p. 552-64.
23. Nguyen, L.N., et al., *Transforming growth factor-beta differentially regulates oval cell and hepatocyte proliferation.* Hepatology, 2007. **45**(1): p. 31-41.
24. Fausto, N. and J.S. Campbell, *The role of hepatocytes and oval cells in liver regeneration and repopulation.* Mech Dev, 2003. **120**(1): p. 117-30.
25. Minguet, S., et al., *A population of c-Kit(low)(CD45/TER119)- hepatic cell progenitors of 11-day postcoitus mouse embryo liver reconstitutes cell-depleted liver organoids.* J Clin Invest, 2003. **112**(8): p. 1152-63.

26. Suzuki, A., et al., *Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells.* Development, 2003. **130**(11): p. 2513-24.
27. Schmelzer, E., E. Wauthier, and L.M. Reid, *The phenotypes of pluripotent human hepatic progenitors.* Stem Cells, 2006. **24**(8): p. 1852-8.
28. Yovchev, M.I., et al., *Novel hepatic progenitor cell surface markers in the adult rat liver.* Hepatology, 2007. **45**(1): p. 139-49.
29. Miyazaki, M., et al., *Improved conditions to induce hepatocytes from rat bone marrow cells in culture.* Biochem Biophys Res Commun, 2002. **298**(1): p. 24-30.
30. Okumoto, K., et al., *Differentiation of bone marrow cells into cells that express liver-specific genes in vitro: implication of the Notch signals in differentiation.* Biochem Biophys Res Commun, 2003. **304**(4): p. 691-5.
31. Chien, C.C., et al., *In vitro differentiation of human placenta-derived multipotent cells into hepatocyte-like cells.* Stem Cells, 2006. **24**(7): p. 1759-68.
32. Kakinuma, S., et al., *Human umbilical cord blood as a source of transplantable hepatic progenitor cells.* Stem Cells, 2003. **21**(2): p. 217-27.
33. Nonome, K., et al., *Human umbilical cord blood-derived cells differentiate into hepatocyte-like cells in the Fas-mediated liver injury model.* Am J Physiol Gastrointest Liver Physiol, 2005. **289**(6): p. G1091-9.
34. Sharma, A.D., et al., *Human cord blood stem cells generate human cytokeratin 18-negative hepatocyte-like cells in injured mouse liver.* Am J Pathol, 2005. **167**(2): p. 555-64.
35. Klug, M.G., et al., *Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.* J Clin Invest, 1996. **98**(1): p. 216-24.
36. Lee, S.H., et al., *Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells.* Nat Biotechnol, 2000. **18**(6): p. 675-9.
37. Hamazaki, T., et al., *Hepatic maturation in differentiating embryonic stem cells in vitro.* FEBS Lett, 2001. **497**(1): p. 15-9.
38. Yarmush, M.L., J.C. Dunn, and R.G. Tompkins, *Assessment of artificial liver support technology.* Cell Transplant, 1992. **1**(5): p. 323-41.
39. Yarmush, M.L., et al., *Hepatic tissue engineering. Development of critical technologies.* Ann N Y Acad Sci, 1992. **665**: p. 238-52.
40. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
41. Amit, M. and J. Itskovitz-Eldor, *Derivation and spontaneous differentiation of human embryonic stem cells.* J Anat, 2002. **200**(Pt 3): p. 225-32.
42. Fair, J.H., et al., *Induction of hepatic differentiation in embryonic stem cells by co-culture with embryonic cardiac mesoderm.* Surgery, 2003. **134**(2): p. 189-96.
43. Dang, S.M., et al., *Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems.* Biotechnol Bioeng, 2002. **78**(4): p. 442-53.
44. Maguire, T., et al., *Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes.* Biotechnol Bioeng, 2006. **93**(3): p. 581-91.
45. Maguire, T., et al., *Control of hepatic differentiation via cellular aggregation in an alginate microenvironment.* Biotechnol Bioeng, 2007.
46. Sharma, N.S., et al., *Sodium butyrate-treated embryonic stem cells yield hepatocyte-like cells expressing a glycolytic phenotype.* Biotechnol Bioeng, 2006. **94**(6): p. 1053-63.
47. Zhou, Q.J., et al., *In vitro differentiation of hepatic progenitor cells from mouse embryonic stem cells induced by sodium butyrate.* J Cell Biochem, 2007. **100**(1): p. 29-42.
48. Teratani, T., et al., *Direct hepatic fate specification from mouse embryonic stem cells.* Hepatology, 2005. **41**(4): p. 836-46.
49. Chinzei, R., et al., *Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes.* Hepatology, 2002. **36**(1): p. 22-9.
50. Choi, D., et al., *In vivo differentiation of mouse embryonic stem cells into hepatocytes.* Cell Transplant, 2002. **11**(4): p. 359-68.
51. Yamamoto, H., et al., *Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application.* Hepatology, 2003. **37**(5): p. 983-93.
52. Jones, E.A., et al., *Hepatic differentiation of murine embryonic stem cells.* Exp Cell Res, 2002. **272**(1): p. 15-22.

53. Hu, A.B., et al., *High-ratio differentiation of embryonic stem cells into hepatocytes in vitro.* Liver Int, 2004. **24**(3): p. 237-45.
54. Jochheim, A., et al., *Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes.* Int J Dev Biol, 2004. **48**(1): p. 23-9.
55. Yamamoto, Y., et al., *Recapitulation of in vivo gene expression during hepatic differentiation from murine embryonic stem cells.* Hepatology, 2005. **42**(3): p. 558-67.
56. Miyashita, H., et al., *Evidence for hepatocyte differentiation from embryonic stem cells in vitro.* Cell Transplant, 2002. **11**(5): p. 429-34.
57. Yamada, T., et al., *In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green.* Stem Cells, 2002. **20**(2): p. 146-54.
58. Gouon-Evans, V., et al., *BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm.* Nat Biotechnol, 2006. **24**(11): p. 1402-11.
59. Heo, J., et al., *Hepatic precursors derived from murine embryonic stem cells contribute to regeneration of injured liver.* Hepatology, 2006. **44**(6): p. 1478-86.
60. Soto-Gutierrez, A., et al., *Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric.* Cell Transplant, 2006. **15**(4): p. 335-41.
61. Soto-Gutierrez, A., et al., *Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes.* Nat Biotechnol, 2006. **24**(11): p. 1412-9.
62. Soto-Gutierrez, A., et al., *Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines.* Nat Protoc, 2007. **2**(2): p. 347-56.
63. Teramoto, K., et al., *Teratoma formation and hepatocyte differentiation in mouse liver transplanted with mouse embryonic stem cell-derived embryoid bodies.* Transplant Proc, 2005. **37**(1): p. 285-6.
64. Leahy, A., et al., *Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation.* J Exp Zool, 1999. **284**(1): p. 67-81.
65. Keller, G.M., *In vitro differentiation of embryonic stem cells.* Curr Opin Cell Biol, 1995. **7**(6): p. 862-9.
66. Shirahashi, H., et al., *Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage.* Cell Transplant, 2004. **13**(3): p. 197-211.
67. Lavon, N., O. Yanuka, and N. Benvenisty, *Differentiation and isolation of hepatic-like cells from human embryonic stem cells.* Differentiation, 2004. **72**(5): p. 230-8.
68. Shiojiri, N. and Y. Sugiyama, *Immunolocalization of extracellular matrix components and integrins during mouse liver development.* Hepatology, 2004. **40**(2): p. 346-55.
69. Sekhon, S.S., et al., *Fibroblast growth factor enriches the embryonic liver cultures for hepatic progenitors.* Am J Pathol, 2004. **164**(6): p. 2229-40.
70. Kamiya, A., T. Kinoshita, and A. Miyajima, *Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways.* FEBS Lett, 2001. **492**(1-2): p. 90-4.
71. Shingu, R., et al., *Expression and distribution of glucose-6-phosphatase catalytic subunit messenger RNA and its changes in the diabetic state.* Res Commun Mol Pathol Pharmacol, 1996. **93**(1): p. 13-24.
72. Han, Y.P., et al., *Essential role of matrix metalloproteinases in interleukin-1-induced myofibroblastic activation of hepatic stellate cell in collagen.* J Biol Chem, 2004. **279**(6): p. 4820-8.
73. Adler, R.R., C.A. Brenner, and Z. Werb, *Expression of extracellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonal carcinoma cells.* Development, 1990. **110**(1): p. 211-20.
74. Kumashiro, Y., et al., *Isolation of hepatocyte-like cells from mouse embryoid body cells.* Transplant Proc, 2005. **37**(1): p. 299-300.
75. Novik, E.I., et al., *Embryoid body-mediated differentiation of mouse embryonic stem cells along a hepatocyte lineage: insights from gene expression profiles.* Tissue Eng, 2006. **12**(6): p. 1515-25.
76. Tsutsui, M., et al., *Characterization of cytochrome P450 expression in murine embryonic stem cell-derived hepatic tissue system.* Drug Metab Dispos, 2006. **34**(4): p. 696-701.
77. Ehashi, T., H. Miyoshi, and N. Ohshima, *Oncostatin M stimulates proliferation and functions of mouse fetal liver cells in three-dimensional cultures.* J Cell Physiol, 2005. **202**(3): p. 698-706.

78. Iwai, M., et al., *Investigation of parenchymal cell differentiation in organotypic slice culture of mouse fetal liver under administration of sodium butyrate.* Cell Biol Toxicol, 2002. **18**(3): p. 147-56.
79. Dunn, J.C., et al., *Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration.* Faseb J, 1989. **3**(2): p. 174-7.
80. Teratani, T., et al., *Long-term maintenance of liver-specific functions in cultured ES cell-derived hepatocytes with hyaluronan sponge.* Cell Transplant, 2005. **14**(9): p. 629-35.
81. Nahmias, Y., F. Berthiaume, and M.L. Yarmush, *Integration of technologies for hepatic tissue engineering.* Adv Biochem Eng Biotechnol, 2007. **103**: p. 309-29.
82. Koide, N., et al., *Continued high albumin production by multicellular spheroids of adult rat hepatocytes formed in the presence of liver-derived proteoglycans.* Biochem Biophys Res Commun, 1989. **161**(1): p. 385-91.
83. Dunn, J.C., R.G. Tompkins, and M.L. Yarmush, *Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration.* Biotechnol Prog, 1991. **7**(3): p. 237-45.
84. Nisoli, E., et al., *Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16507-12.
85. Leary, S.C. and E.A. Shoubridge, *Mitochondrial biogenesis: which part of "NO" do we understand?* Bioessays, 2003. **25**(6): p. 538-41.
86. Peunova, N. and G. Enikolopov, *Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells.* Nature, 1995. **375**(6526): p. 68-73.
87. Magrinat, G., et al., *Nitric oxide modulation of human leukemia cell differentiation and gene expression.* Blood, 1992. **80**(8): p. 1880-4.
88. Bloch, W., et al., *Nitric oxide synthase expression and role during cardiomyogenesis.* Cardiovasc Res, 1999. **43**(3): p. 675-84.
89. Lin, J., et al., *PGC-1beta in the regulation of hepatic glucose and energy metabolism.* J Biol Chem, 2003. **278**(33): p. 30843-8.
90. Naiki, T., et al., *Analysis of gene expression profile induced by hepatocyte nuclear factor 4alpha in hepatoma cells using an oligonucleotide microarray.* J Biol Chem, 2002. **277**(16): p. 14011-9.
91. Houten, S.M. and J. Auwerx, *PGC-1alpha: turbocharging mitochondria.* Cell, 2004. **119**(1): p. 5-7.
92. Liang, H. and W.F. Ward, *PGC-1alpha: a key regulator of energy metabolism.* Adv Physiol Educ, 2006. **30**(4): p. 145-51.
93. Kamiya, A., F.J. Gonzalez, and H. Nakauchi, *Identification and differentiation of hepatic stem cells during liver development.* Front Biosci, 2006. **11**: p. 1302-10.
94. Znoyko, I., et al., *Expression of oncostatin M and its receptors in normal and cirrhotic human liver.* J Hepatol, 2005. **43**(5): p. 893-900.
95. Kumashiro, Y., et al., *Enrichment of hepatocytes differentiated from mouse embryonic stem cells as a transplantable source.* Transplantation, 2005. **79**(5): p. 550-7.
96. Behnia, K., et al., *Xenobiotic metabolism by cultured primary porcine hepatocytes.* Tissue Eng, 2000. **6**(5): p. 467-79.
97. Roy, P., et al., *Effect of flow on the detoxification function of rat hepatocytes in a bioartificial liver reactor.* Cell Transplant, 2001. **10**(7): p. 609-14.

FIGURES

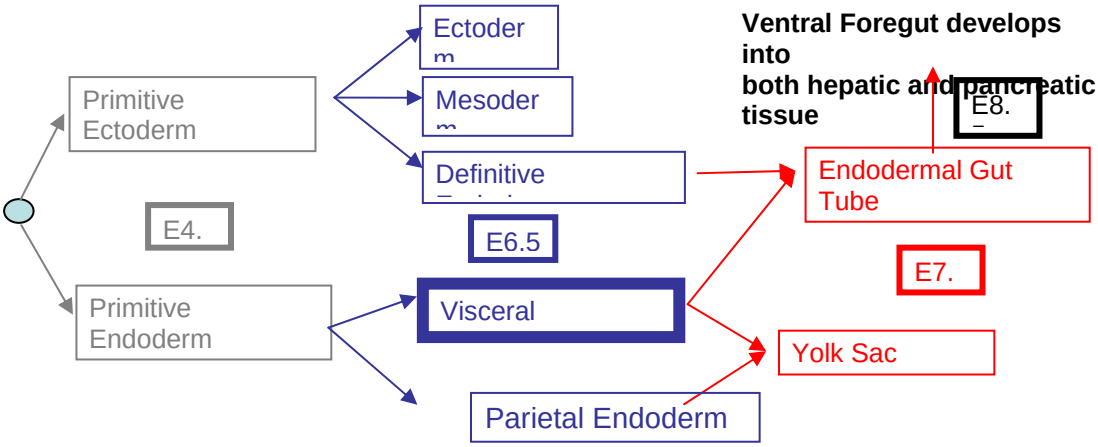


Figure1.1 Schematic Representation of InVivo Lineage Formation. The three adult germ layers are derived from the primitive ectoderm at embryonic day 6.5. At the same time, the visceral and parietal endoderm are formed from the primitive endoderm. The visceral endoderm exhibits markers similar to the differentiating liver but does not differentiate into the adult liver.

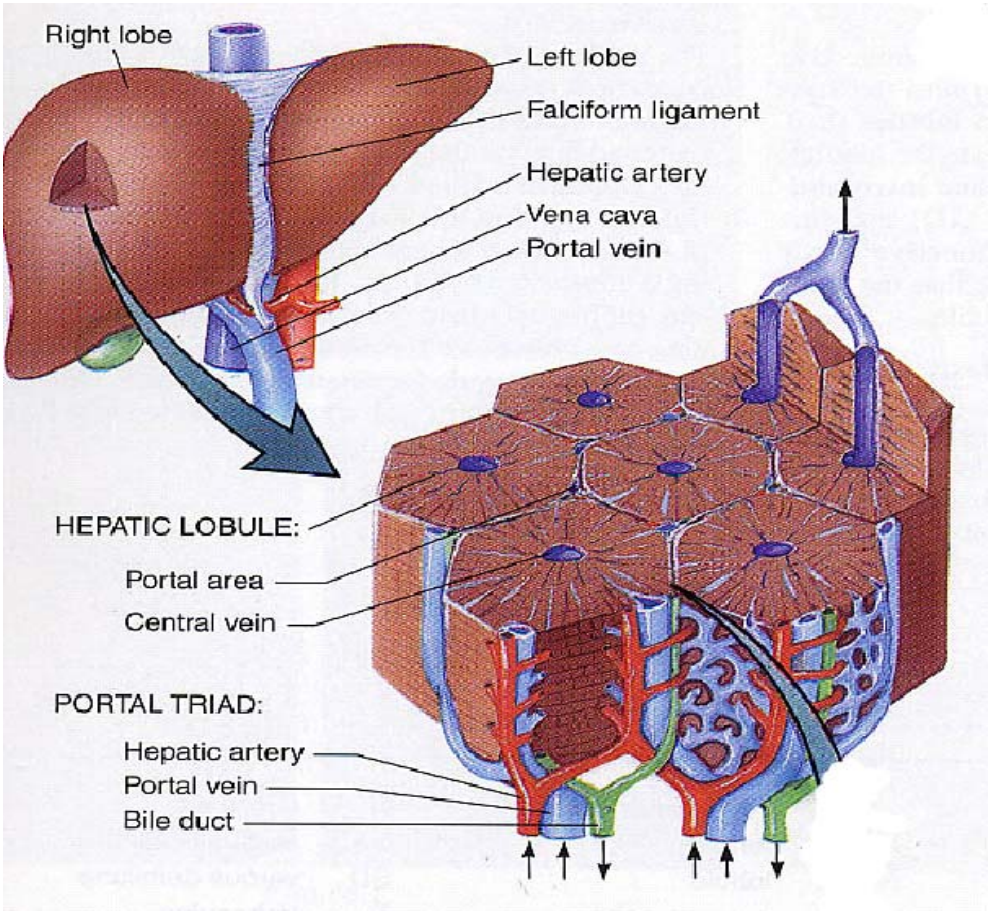


Figure1.2 Schematic Representation of Liver/Hepatocyte Structure. The liver is divided into hepatic lobules which are centered on a central draining vein. Each lobule is comprised of polygonal shaped hepatocytes organized into unicellular plates.

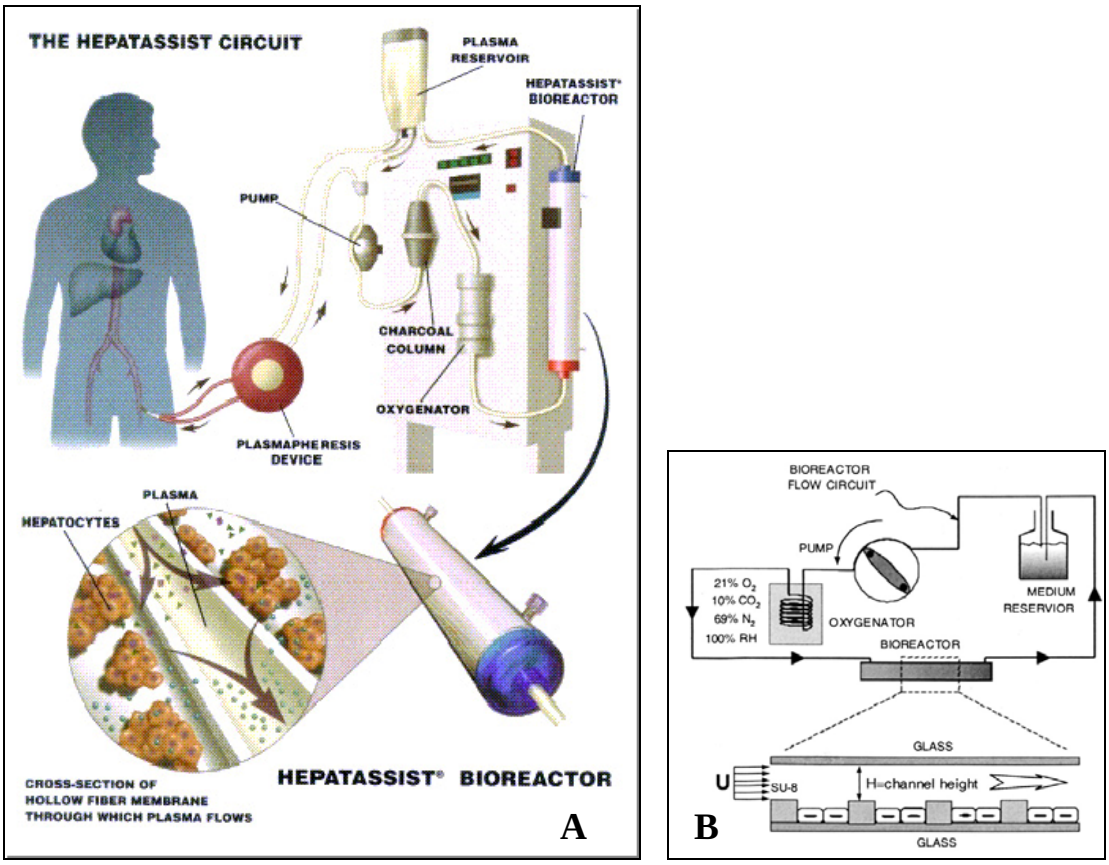


Figure 1.3 Bioreactor Designs A) Schematic of Hepatassist Bioreactor Circuit. **(**Schematic for the future application of BAL circuit to detoxify blood**.)** Patient plasma, which is separated from the blood, is pumped through an oxygenator and subsequently through the hollow fiber design bioreactor where it gets detoxified by the embedded hepatocytes. The plasma is then reintroduced to whole blood and re-circulated. B) Schematic of grooved bioreactor and profusion circuit

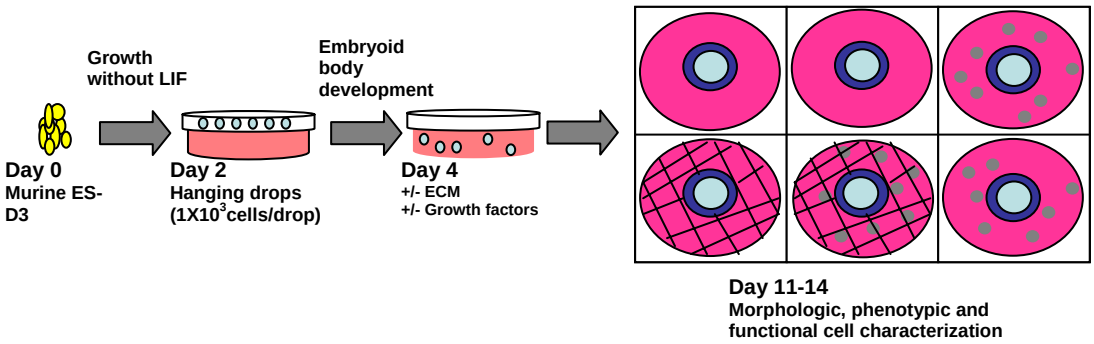
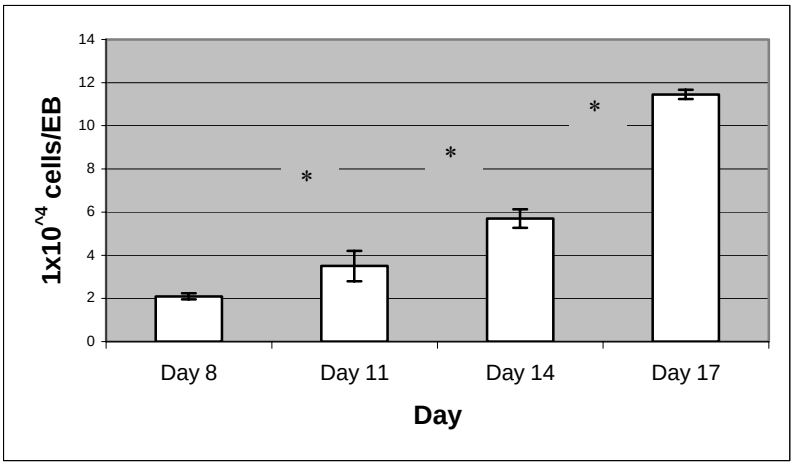


Figure 2.1 Schematic Representation of Hanging Drop Protocol. Hanging drops are plated on Day 0 at a density of 1000 cells/ drop. They are left hanging for 48 hrs. after which they are washed off and suspended in IMDM for an additional 48 hrs. The EBs are then plated on C and tissue culture polystyrene and growth factors are added.

A



B

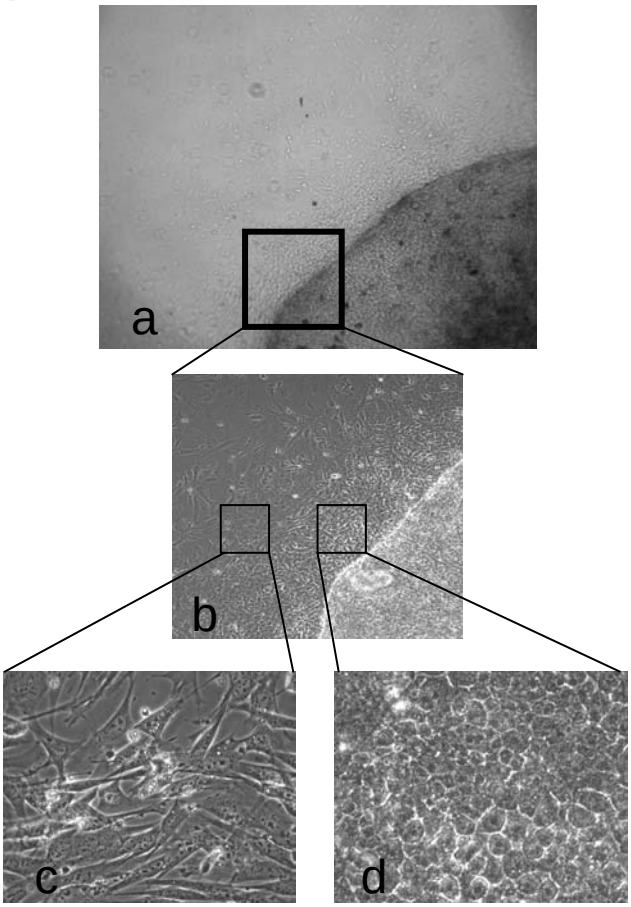


Figure 2.2 EB Size and Morphology Characterization. A) EB cell number was assessed by counting total cells per EB dissociated following trypsinization. Asterisk (*) indicates time points at which the central condensed core was not dissociated by trypsin. B) a) 4x phase contrast image of EB core and migrating outer cells b) 10x phase contrast image of core border with monolayer cells which have migrated out of the core. c) 40x phase contrast image of cells of an elongated morphology located on the outer edge of the growing EB. d) 40x phase contrast image of densely packed cells located in between the core and outer region of elongated cells. All images where acquired on Day 17 post differentiation induction.

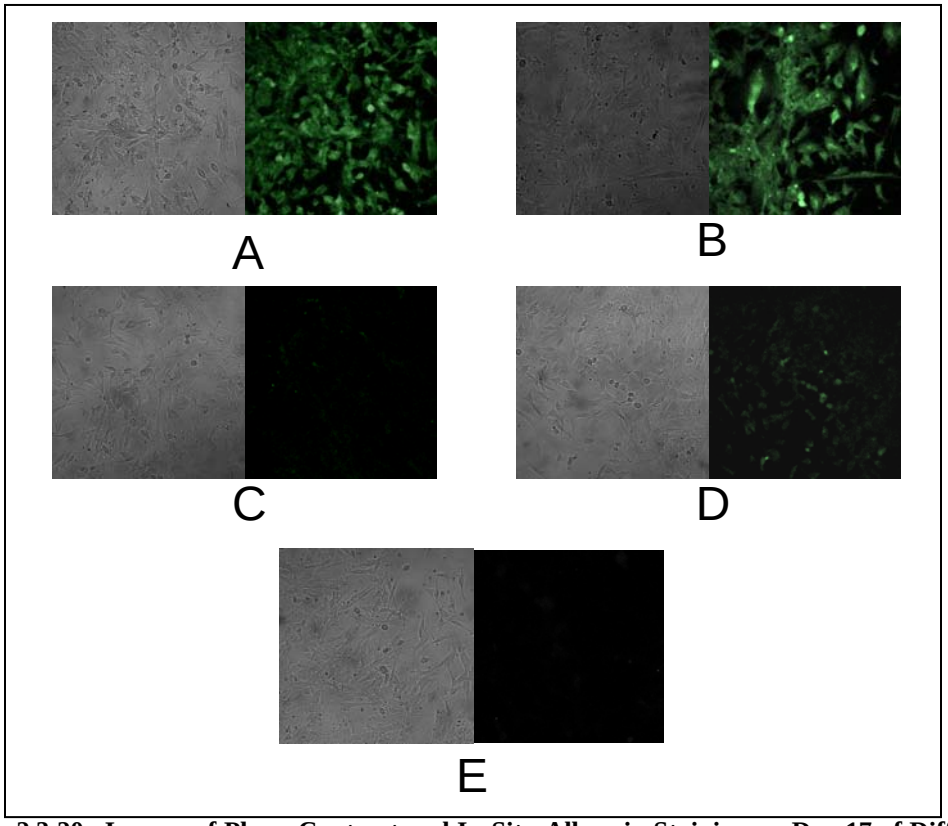
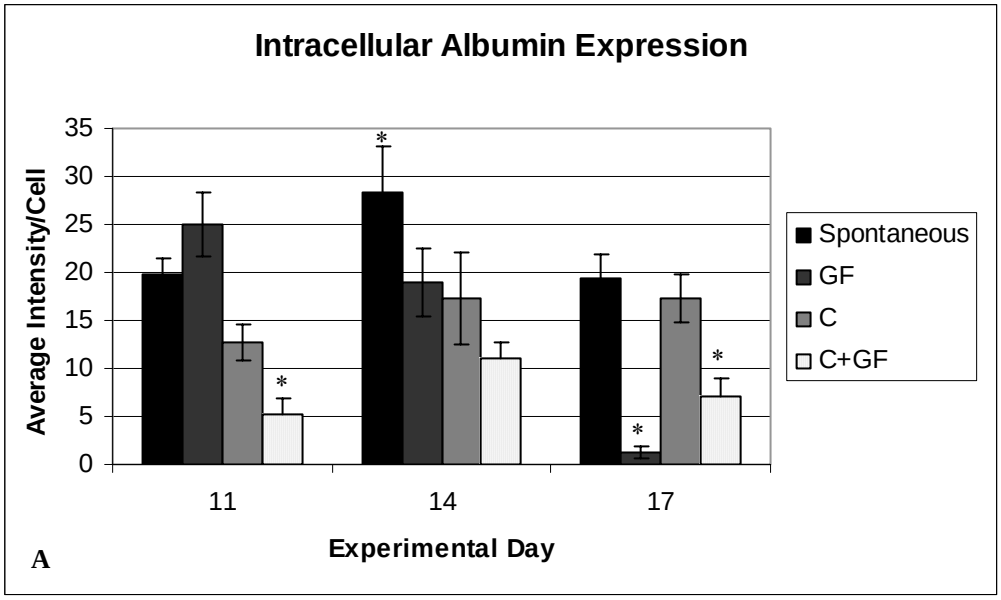


Figure 2.3 20x Images of Phase Contrast and In-Situ Albumin Staining on Day 17 of Differentiation. Differentiated EB's in the four culture conditions were fixed in place with paraformaldehyde and stained with an anti-albumin antibody. The image (phase contrast, left and fluorescent, right) were captured with a computer interfaced Zeiss inverted microscope. (A) Spontaneous (B) C (C) GF (D) C+GF (E) ES control.



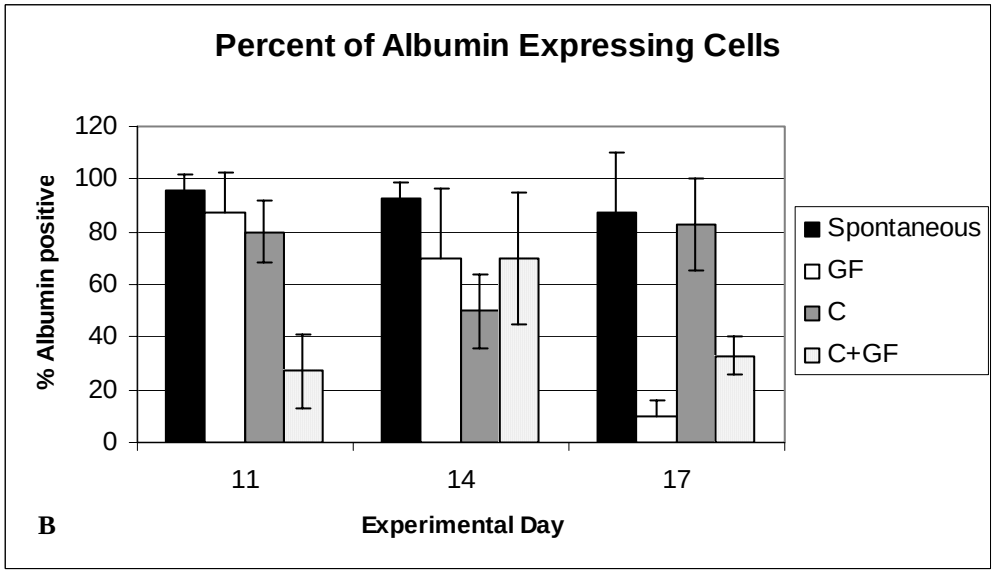


Figure 2.4 Intracellular Albumin Expression. A) Time course of albumin expression levels in the four conditions. Undifferentiated ES cells were used as a negative control (average intensity (0). Each data point represents the mean of a sample size of thirty cells from the monolayer, where the mean average intensity value across the entire cell is used in the average of the 30 cells and error bars represent standard error of the mean of 30 cells. Asterisk (*) indicates statistically significant differences ($p<0.05$) from all other conditions on that day. B) Time course of the percentage of outer region EB cells expressing albumin in the four conditions. Each data point represents the % of cells with an intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control.

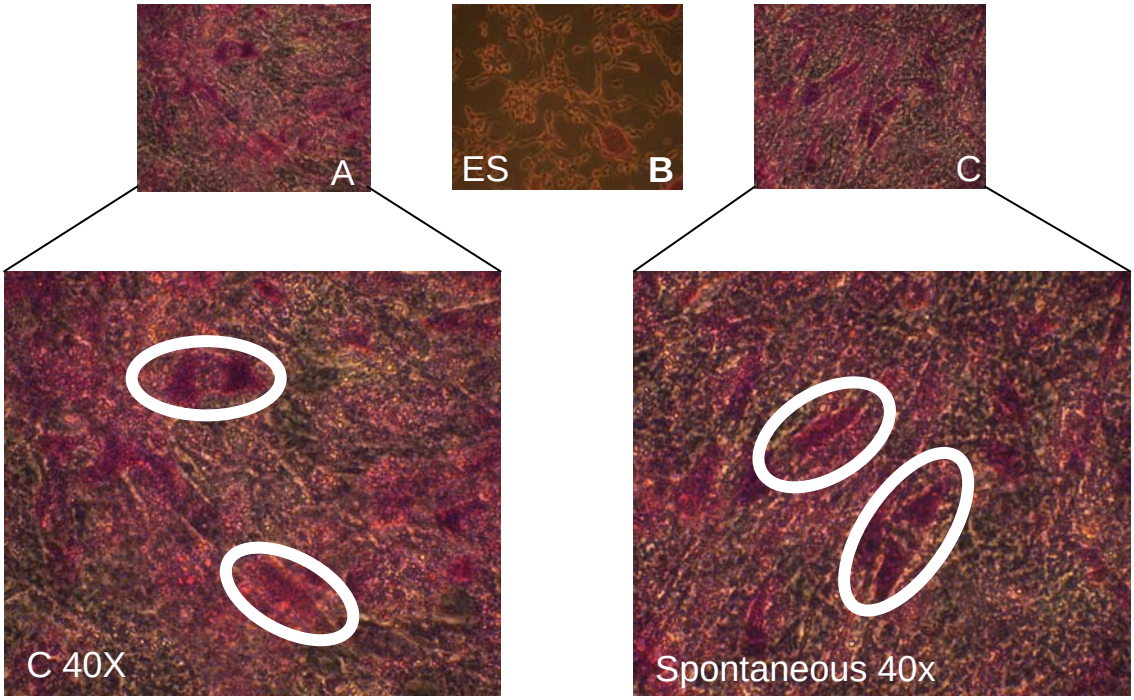
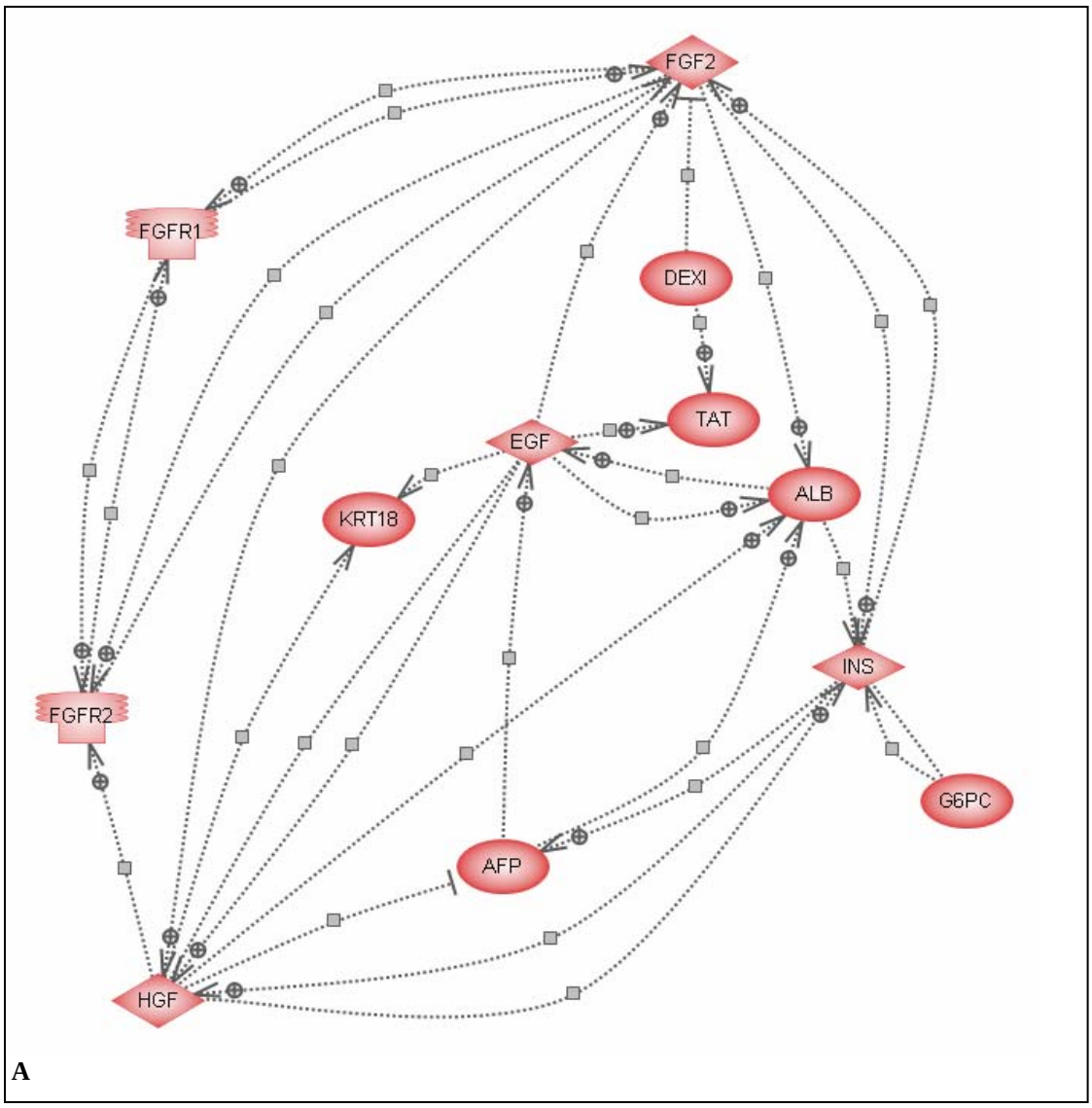


Figure 2.5 40x Images of Phase Contrast, In-Situ Glycogen Staining on Day 17 of Differentiation. Differentiated EB's in the C and NC culture conditions were fixed in place with paraformaldehyde and stained for glycogen storage. The images were captured with an Olympus inverted microscope. (A) C (B) ES control (C) Spontaneous



A

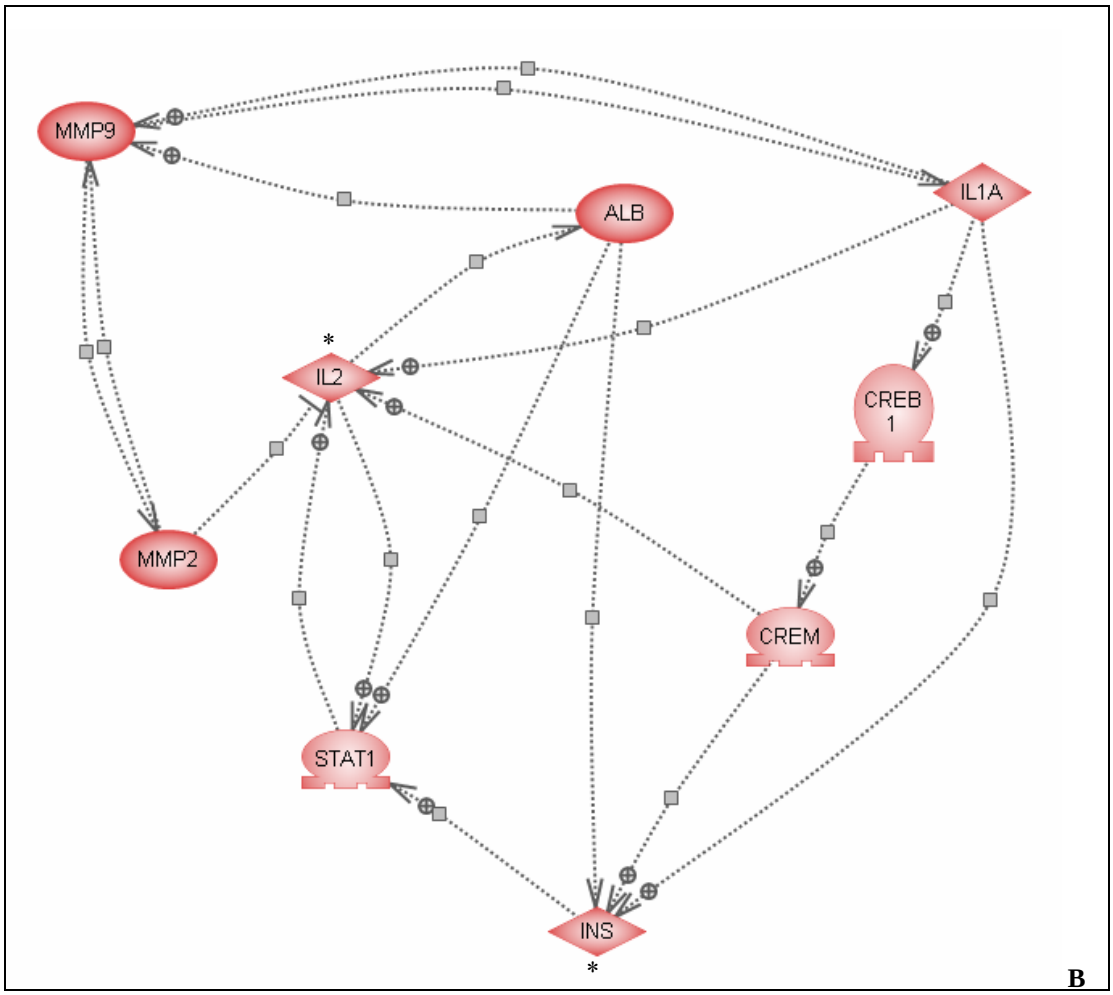
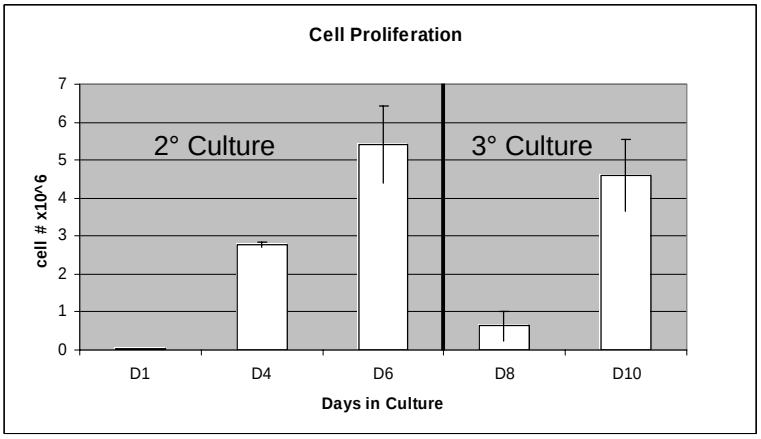
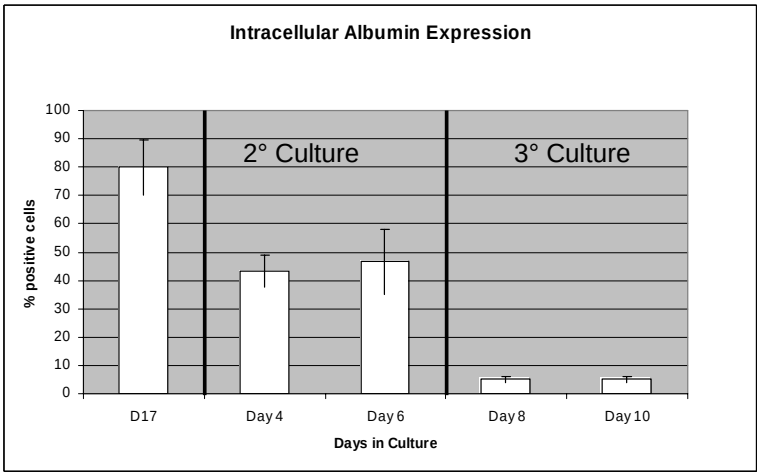


Figure 2.6 Pathway Assist Generated Pathways. The pathways were generated utilizing the shortest path approach to link differentially expressed genes. A) Input genes were regulated in the spontaneous condition only. Asterisk (*) Indicates genes identified and inserted by Pathway Assist. B) Input genes were regulated in the C+GF and C conditions. Asterisk (*) Indicates genes identified and inserted by Pathway Assist.

3.1A)



3.1B)



3.1C)

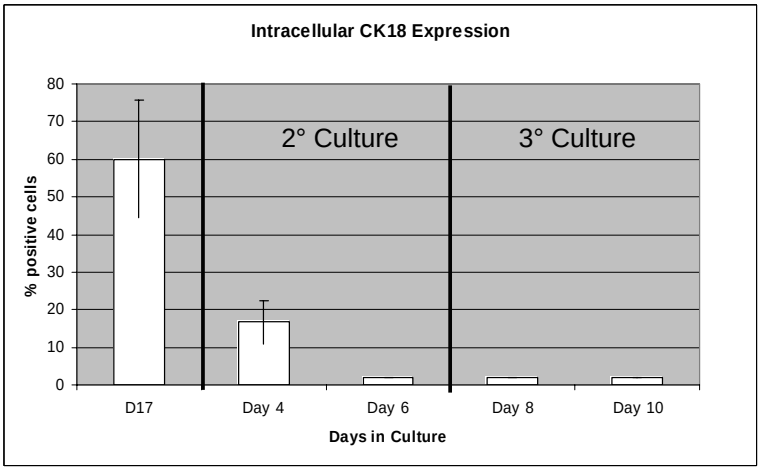
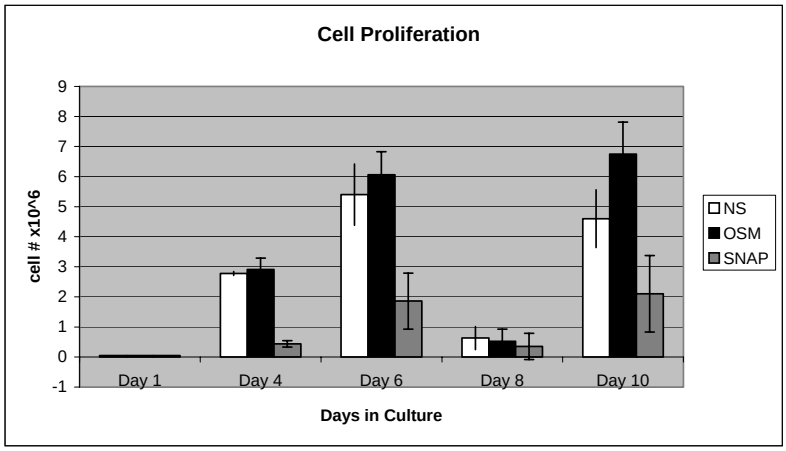


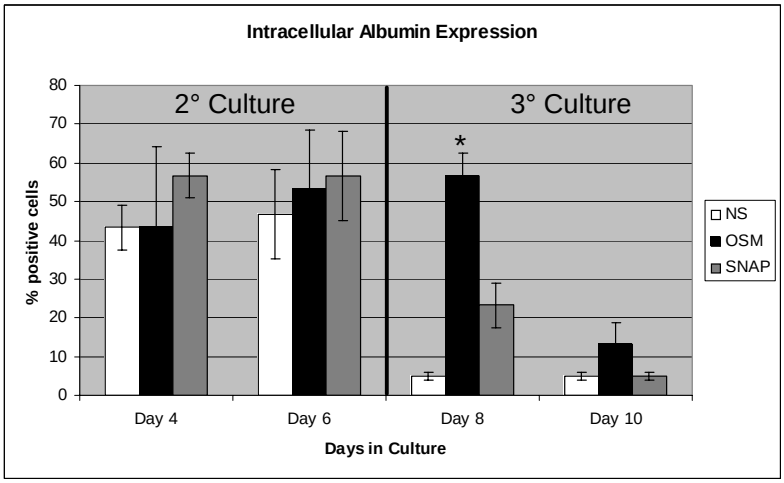
Figure 3.1. Non Supplemented Secondary Culture Characterization. A) Cell number was assessed by counting total cells dissociated following trypsinization. Cells were plated into tertiary culture at $5x10^4$ cells per well at Day 6 B) Time course of the percentage of cells expressing albumin in polystyrene secondary

culture. Each data point represents the % of cells with an intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control. C) Time course of the percentage of cells expressing CK18 in polystyrene secondary culture. Each data point represents the % of cells with a normalized intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control.

3.2A)



3.2B)



3.2C)

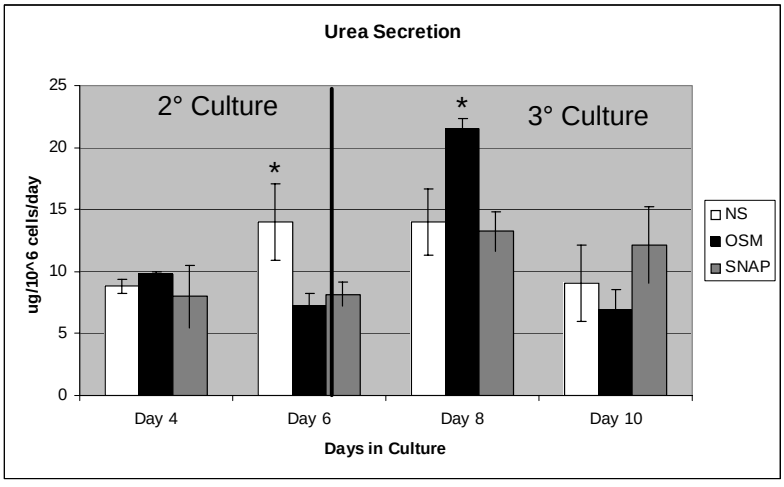
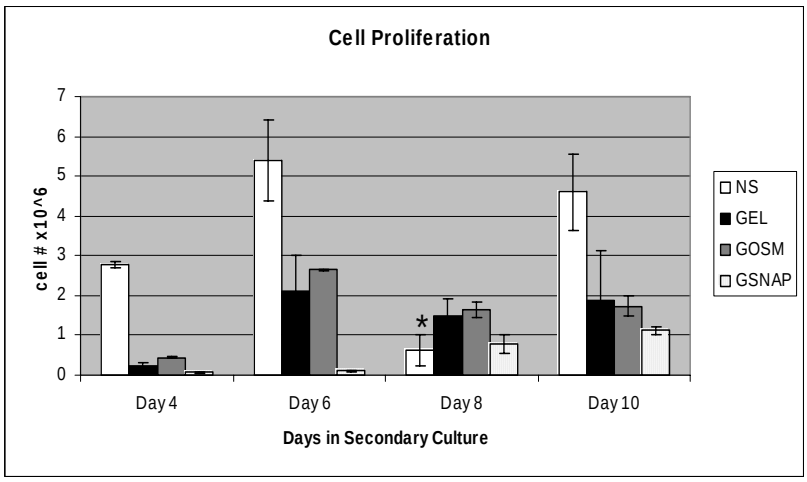
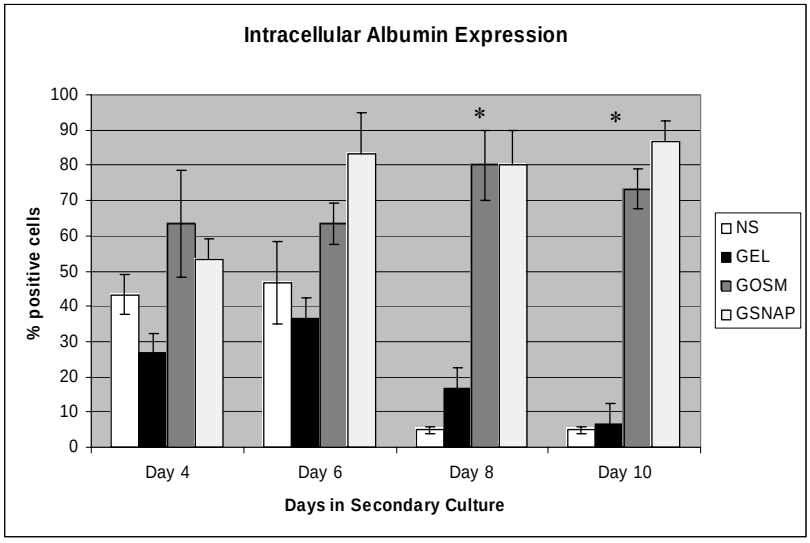


Figure 3.2. OSM and SNAP Supplemented Secondary Culture Characterization. A) Cell number was assessed by counting total cells dissociated following trypsinization. Cells from the three conditions were plated into tertiary culture at $5x10^4$ cells per well at Day 6 B) Time course of the percentage of cells expressing albumin in polystyrene secondary culture. Each data point represents the % of cells with an intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control. Asterisk (*) indicates statistically significant differences ($p<0.05$) from other conditions on that day. C) Time course of urea secretion rates in the supplemented conditions. The average of three experiments is presented. All values were statistically significant as compared to the ES control. Asterisk (*) indicates statistically significant differences ($p<0.05$) from other conditions on that day.

3.3A)



3.3B)



3.3C)

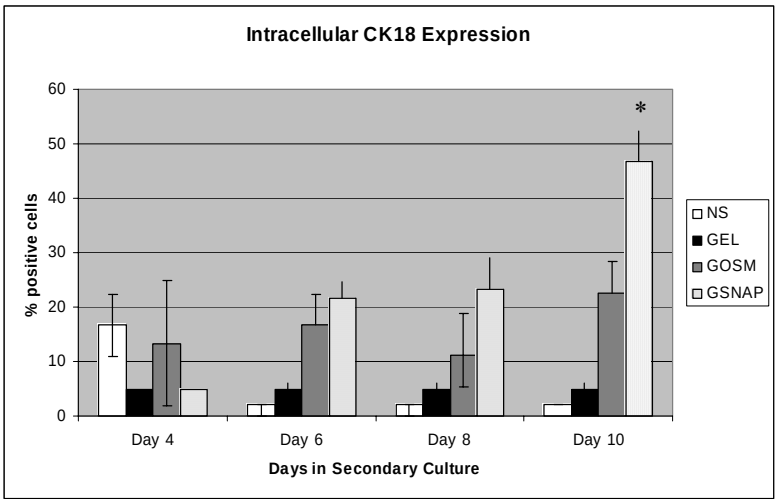
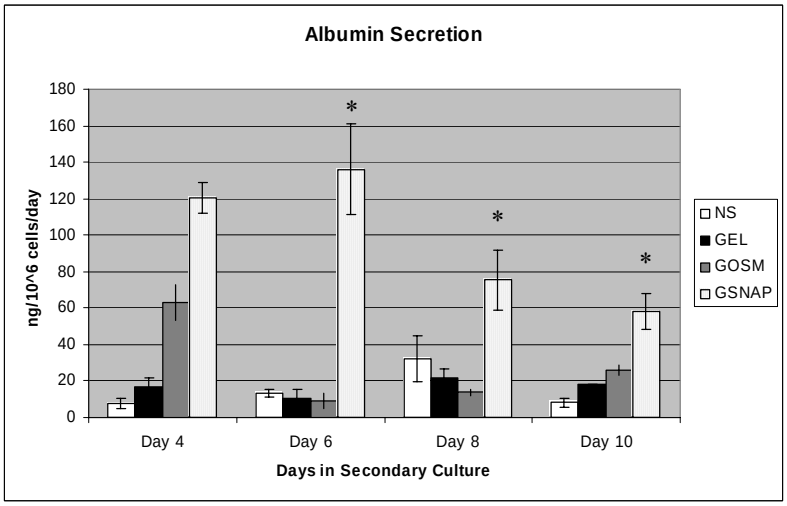


Figure 3.3. Collagen Sandwich Secondary Culture Characterization. A) Cell number was assessed by counting total cells dissociated following trypsinization. Cell number in all GEL conditions was assessed by counting the total number of cells dissociated following collagenase digestion and trypsinization. Asterisk (*) indicates time point at which the NS cells were passed to $5x10^4$ cells per well. B) Time course of the percentage of cells expressing albumin in polystyrene secondary culture. Each data point represents the % of cells with an intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control. Asterisk (*) indicates statistically significant differences ($p<0.05$) from NS and GEL conditions on that day. C) Time course of the percentage of cells expressing CK18 in the sandwich culture conditions. Each data point represents the % of cells with an intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control. Asterisk (*) indicates statistically significant differences ($p<0.05$) from all other conditions on that day.

3.4A)



3.4B)

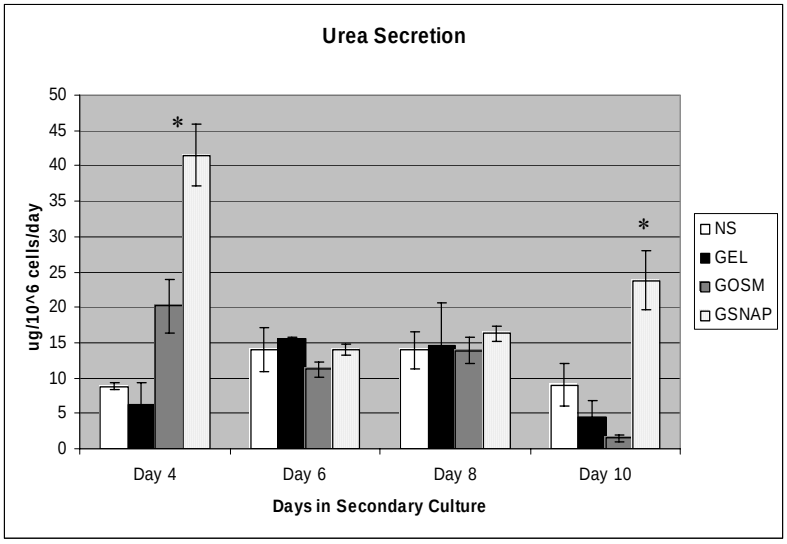


Figure 3.4. **Albumin and Urea Secretion Rates in Sandwich Culture.** A) Time course of ALB secretion rates in the sandwich culture conditions. The average of three experiments is presented. All values were statistically significant as compared to the ES control. Asterisk (*) indicates statistically significant differences ($p<0.05$) from all other conditions on that day. B) Time course of urea secretion rates in the sandwich culture conditions. The average of three experiments is presented. All values were statistically significant as compared to the ES control. Asterisk (*) indicates statistically significant differences ($p<0.05$) from all other conditions on that day.

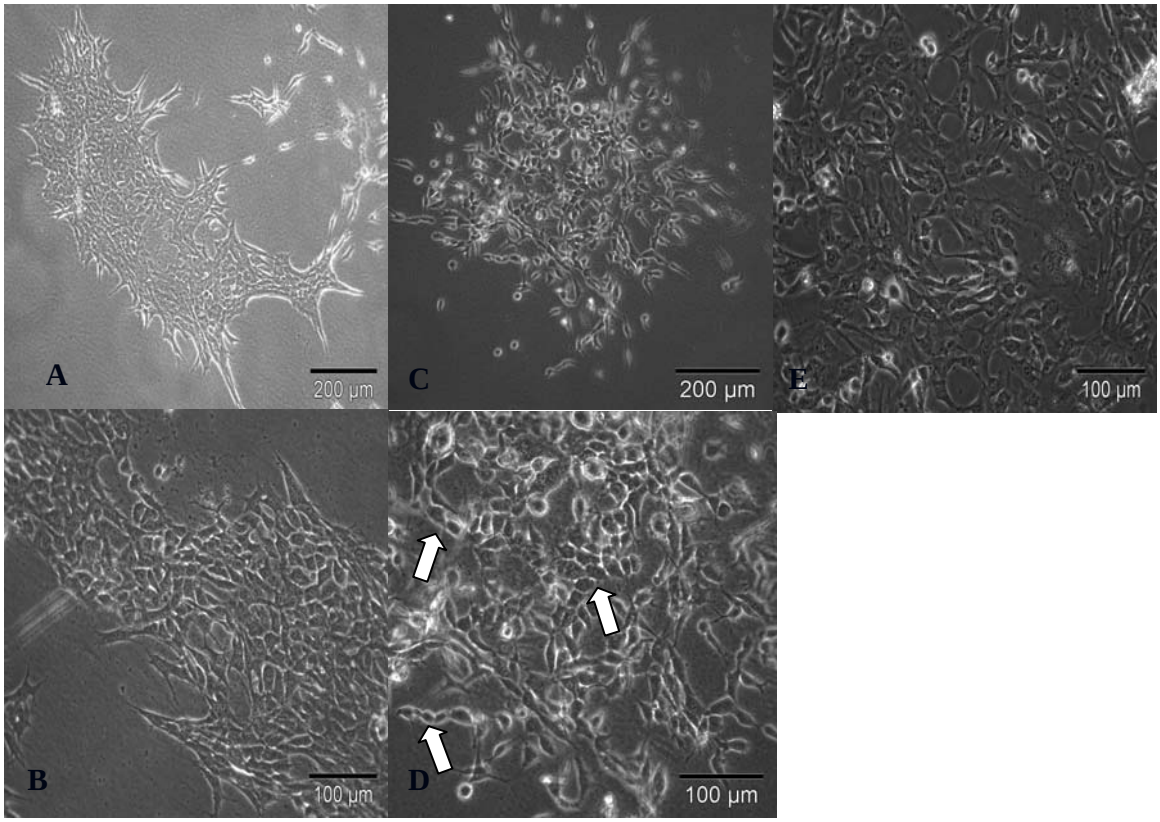
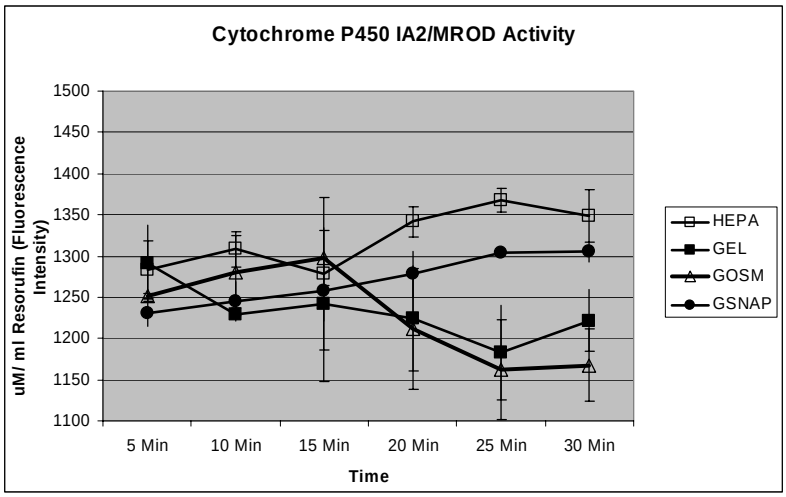
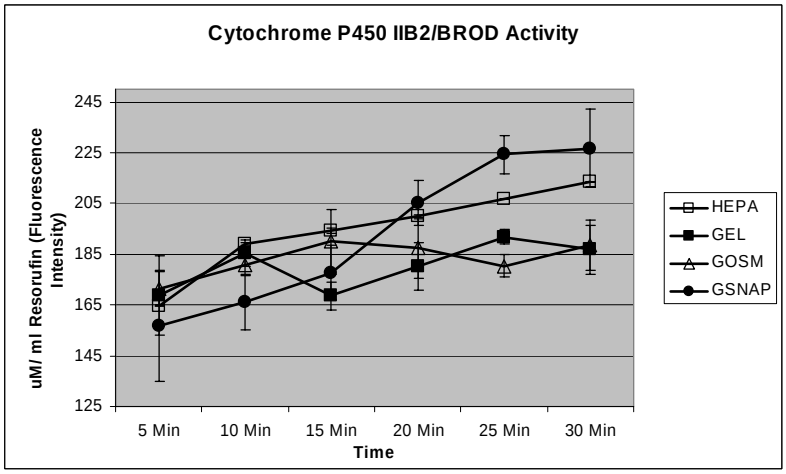


Figure 3.5. Cellular Morphologies. A) 10x magnification, phase contrast image of cells in the GEL condition. Cells with similar morphologies were observed in all GEL conditions B) 20x magnification, phase contrast image of cells described in A. C) 10x magnification, phase contrast image of GSNAP cells in a non confluent, loosely connected environment. D) 20x magnification, phase contrast image of GSNAP cells were greater than 95% of cells are in groups of round or square cells indicated by white arrows. E) 20x magnification, phase contrast image of NG cells. Cells with similar morphologies were observed in all non GEL conditions.

3.6A)



3.6B)



3.6C)

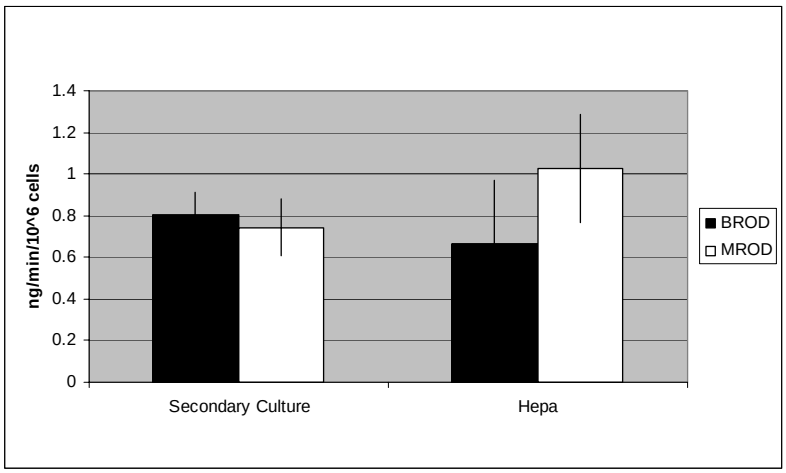


Figure 3.6. Cytochrome P450 detoxification All graphs represent cells which have been in secondary culture for 10 days. Hepa 1-6 were used as a positive control. A) MROD activity is measured every five minutes via metabolism of methoxyresorufin to resorufin. Increases in resorufin concentration indicate activity. B) BROD activity is measured every five minutes via metabolism of methoxyresorufin to resorufin. Increases in resorufin concentration indicate activity. C) Averaged rates of production of MROD and BROD based on total cell number.

List of Tables

Conditions	Upregulated	Downregulated	Total
Spontaneous	1410	1663	3073
+GF	996	1082	2078
C	626	511	1137
C+GF	1261	792	2053

Table 2.1 Effects of the Four Culture Conditions on Differential Gene Expression. Microarray analysis of differentially expressed genes 17 days after the start of differentiation. Expression levels of differentiated cells were compared to expression profiles of ES cells. Here, all genes which were upregulated or downregulated as compared to ES cells ($p<0.1$) are shown for the four conditions.

Gene	Spontaneous	GF	C	C+GF
Cytochrome P450's				
CYP 1B1	**0.891**	N/S	N/S	N/S
CYP 11A1	**-0.821**	N/S	N/S	N/S
CYP 19A1	**0.414**	N/S	N/S	N/S
CYP 2E1	**0.385**	N/S	**1.405**	N/S
CYP 2J6	**1.098**	N/S	N/S	N/S
CYP 2S1	**-0.567**	N/S	N/S	N/S
CYP 27B1	**0.397**	**0.281**	N/S	N/S
CYP 3A13	**-0.346**	N/S	N/S	N/S
CYP 3A25	**1.981**	**1.59**	N/S	N/S
CYP 46A1	**-0.18**	N/S	N/S	N/S
CYP 51	**0.522**	**0.489**	**-0.377**	**-0.7**
Cytokeratin 18	**1.146**	N/S	N/S	N/S
Alcohol dehydrogenase 1	**0.919**	**1.191**	N/S	N/S
Aldehyde dehydrogenase family 3A2	**1.679**	**0.985**	N/S	N/S
Cadherin 17	**0.755**	N/S	N/S	N/S
Transthyretin	**0.551**	N/S	**0.769**	N/S

Table 2.2 Representative Genes Differentially Expressed in at Least One of the Four Differentiation Conditions. All numbers represent the *log2(mean cy3/cy5)* of that specific gene. N/S is representative of all non-significant differences ($p>0.1$) in the *log2(mean cy3/cy5)* ratio.

Gene	Spontaneous	GF	C	C+GF
Growth Factors				
Hepatocyte growth factor	**0.595**	N/S	N/S	N/S
Fibroblast growth factor 2	**0.273**	N/S	N/S	N/S
Fibroblast growth factor 10	N/S	**0.99**	N/S	**0.81**
Fibroblast growth factor 17	**0.913**	**-1.11**	N/S	**-0.795**
Fibroblast growth factor receptor 1	**0.864**	N/S	N/S	N/S
Fibroblast growth factor receptor 2	**1.367**	**1.456**	N/S	N/S
Matrix Proteins				
Fibronectin 1	**1.107**	**0.574**	N/S	N/S
Laminin gamma 3	**-1.031**	**-1.059**	N/S	N/S
Laminin, alpha 4	**0.358**	N/S	N/S	N/S
Laminin, beta 3	**1.092**	**0.955**	N/S	**-0.809**
Laminin, gamma 2	**-0.644**	**-0.642**	N/S	**0.598**
Procollagen, type II, alpha 1	N/S	N/S	**-0.666**	N/S
Procollagen, type IV, alpha 3	**-0.447**	N/S	N/S	**1.184**
Procollagen, type IV, alpha 4	**-0.269**	N/S	N/S	N/S
Procollagen, type IV, alpha 6	**-2.057**	**-2.044**	N/S	N/S
Procollagen, type V, alpha 2	**0.837**	0	N/S	N/S
Procollagen, type VI, alpha 3	**0.688**	**0.374**	N/S	N/S
Procollagen, type X, alpha 1	**-1.281**	N/S	N/S	N/S
Procollagen, type XI, alpha 1	**-0.417**	N/S	N/S	N/S
Procollagen, type XI, alpha 2	N/S	N/S	**0.787**	**0.727**
Adhesion Proteins				
Integrin alpha 2	N/S	N/S	**0.76**	N/S
Integrin alpha 3	**-1.307**	**-1.114**	N/S	**0.976**
Integrin beta 4	**0.91**	**1.005**	N/S	N/S
Integrin beta 5	N/S	**-0.738**	N/S	N/S
Integrin beta 6	N/S	**0.64**	N/S	**-0.782**
Cadherin 6	**0.78**	**0.78**	N/S	**0.428**
Cadherin 8	**1.734**	N/S	N/S	N/S
Cadherin 9	N/S	N/S	N/S	**0.874**
Cadherin 17	**0.755**	N/S	N/S	N/S
Cadherin 22	**-0.213**	**0.778**	N/S	N/S

Table 2.3 Representative Differentially Expressed Mechanistic Genes. All numbers represent the *log2(mean cy3/cy5)* of that specific gene. N/S is representative of all non significant differences (p>.1) in the *log2(mean cy3/cy5)* ratio.

Gene	Spontaneous	GF	C	C+GF
Interleukin 1 alpha	N/S	N/S	**0.440**	**0.920**
Interleukin 6	**-0.470**	N/S	N/S	N/S
Tumor necrosis factor	**-0.980**	**-0.922**	N/S	N/S
CAMP responsive element modulator	N/S	N/S	N/S	**0.694**
AP-1, gamma 1 subunit	N/S	N/S	N/S	**1.281**
X-box binding protein 1	**-0.350**	N/S	N/S	N/S
STAT 1	N/S	N/S	N/S	**1.732**
Protein inhibitor of activated STAT 3	N/S	N/S	**0.937**	**0.877**
Matrix metalloproteinase 2	N/S	N/S	N/S	**5.132**
Matrix metalloproteinase 9	N/S	N/S	N/S	**0.868**

Table 2.4 Acute Phase Response Genes. All numbers represent the *log2(mean cy3/cy5)* of that specific gene. N/S is representative of all non significant differences (p>.1) in the *log2(mean cy3/cy5)* ratio.

Day 17 Culture Expressed Gene	Cell Lineage	Culture Condition
Neural Cell Adhesion Molecule	Neuron	Spontaneous, +GF
Neurofilament	Neuron	Spontaneous
Actinin	Cardiac	+Collagen
Calcium Channel alpha unit	Cardiac	+ Collagen
GATA binding protein 5	Cardiac	+ Collagen
Spectrin beta 2	Cardiac	+ Collagen

Table 2.5: Representative Upregulated Genes of Neuron and Cardiac Tissue Lineages. All numbers represent the *log2(mean cy3/cy5)* of that specific gene. N/S is representative of all non significant differences (p>.1) in the *log2(mean cy3/cy5)* ratio.

	NS	SNAP	OSM
Intracellular ALB	+	+	+
CK18	+	-	-
Urea	+	+	+
ALB Secretion	-	-	-
CYP450	-	-	-
Glycogen	-	-	-

Table 3.1. Function Summary for Supplemented Cultures. A (+) represents at least one time point of significant expression or secretion function in the three conditions. A (-) represents functions which were absent on all experimental days.

CURRICULUM VITAE

Eric I. Novik

EDUCATION:

2002 – June 2007 **Ph.D., Biomedical Engineering**
Department of Biomedical Engineering
Rutgers, The State University of New Jersey

1998 -May 2002 **B.S., Biomedical Engineering**
Department of Biomedical Engineering
Rutgers, The State University of New Jersey

PUBLICATIONS:

Novik, E.I., Maguire, T.J., Schloss, R., Yarmush, M.L. Embryoid body mediated differentiation of mouse embryonic stem cells along a hepatocyte lineage: insights into kinetic and gene expression profiles. **Tissue Eng** 2006; 12: 1515-25.

Maguire, T.J., **Novik E.I.,** Schloss, R., Yarmush, M.L. Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes. **Biotechnol Bioeng** 2006; 93: 581-91.

Maguire, T.J., Davidovich, A., **Novik, E.,** Sharma, N., Wallenstein, E., Schloss, R., Yarmush, M.L. Characterization of encapsulation as a method for the hepatic differentiation of embryonic stem cells. **Biotechnol Bioeng** 2007; March 27

N. S. Sharma, E. J. Wallenstein, T. Maguire, **E. Novik**, R. Schloss, M. G. Ierapetritou, M. L. Yarmush. S-Nitrosoacetylpenicillamine upregulates mitochondrial and differentiated function in hepatocyte-like cells derived from embryonic stem cells. **Biotechnol Bioeng** (submitted)

Novik, E.I., Maguire, T.J., Schloss, R., Yarmush, M.L. Secondary culture systems for maximization of hepatic differentiation of embryoid bodies. (in preparation)