THREE-DIMENSIONAL CULTURE OF FETAL LIVER CELLS

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Presented to the Faculty of the Graduate School
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In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
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This thesis explored the influence of culture environment on proliferation and hepatic differentiation of fetal liver cells. Specifically, signals provided by soluble factors and culture substrates were examined. Comparison of a variety of culture medium conditions suggested that hepatocyte growth factor (HGF) is required throughout the culture period to induce the differentiation of liver stem cells into hepatic precursors, and to ensure the continual proliferation of stem cells and their progeny. On the other hand, timing of the addition of maturation-inducing factors such as oncostatin M (OSM) determines the kinetics of cell maturation. In the presence of HGF, adding OSM from the beginning results in concurrent growth and differentiation. In addition, the constant presence of protective agents such as ascorbic acid helps to enhance cell survival, leading to improved cell expansion and hepatocyte functions. Based on these findings, a modified culture medium was developed to achieve both optimal growth and differentiation. Cell expansion in this medium was about 2 fold of that under other tested conditions, whereas the specific albumin secretion rate was 2 - 3 times of the maximal values obtained with other conditions. Subsequently, characterization of fetal liver cells cultured on a variety of three-dimensional (3D) scaffolds, including decellularized liver matrices, collagen scaffolds, and poly(lactic-co-glycolic acid) (PLGA) scaffolds, showed that the cellular responses to soluble signals are modulated by culture substrates. Experiments using scaffolds prepared from blends of PLGA(50:50) and polycaprolactone (PCL) further suggested that cell expansion requires rigid and porous scaffolds, whereas differentiation is
enhanced on more pliable substrates. Together, these results demonstrated that the \textit{in vitro} behavior of fetal liver cells is regulated by both the soluble and physical cues present in their microenvironment. Furthermore, cell growth and differentiation are influenced not only by the regulatory factors that are present, but also by the timing of their addition. These findings would help to develop a culture system that employs dynamic integration of biochemical and substrate-related signals to control the growth and differentiation kinetics of fetal liver cells. They may also provide a foundation for developing technologies for applications of other liver stem cells and progenitors.
BIOGRAPHICAL SKETCH

Lichuan Qian was born in Chengdu, China. She received a B.E. in Biomedical Engineering from the Huazhong University of Science and Technology, China and a M.S. in Microbial Engineering from the University of Minnesota, Twin Cities. Before entering the Ph.D. program in Chemical Engineering at Cornell University, she worked on the research and development of cell therapy and medical devices for cancer vaccines and chronic viral infections in a biotech company.
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1.1 LIVER FUNCTIONS

The liver, the largest gland in the body, is vital to the body’s metabolism and detoxification activities. The liver’s functions are carried out primarily by its parenchymal cells – hepatocytes, which perform an astonishingly large number of activities. Below is a brief review of the key functions of the liver.

_Amino acid metabolism_

The liver plays a central role in amino acid metabolism. Amino acids in the plasma are removed by hepatocytes for protein synthesis or energy production. In addition, the liver is the principal site for the synthesis of non-essential amino acids and for the re-amination of most essential amino acids. Furthermore, it is a major site for amino acid breakdown. The majority of the nitrogen released from amino acid catabolism, in the form of highly toxic ammonia, is converted to urea by hepatocytes via the urea cycle that occurs almost exclusively in the liver.

_Protein synthesis_

Proteins synthesized by hepatocytes are predominantly plasma proteins, and account for approximately 15% of the body’s total protein production. Albumin, the major plasma protein, is synthesized almost exclusively by the liver. In addition, hepatocytes are responsible for the production of transport proteins such as transferrin, all but factor VIII of the coagulation cascade components, many of the proteins
involved in fibrinolysis, most of the complement system proteins, and numerous protease inhibitors.

**Carbohydrate metabolism**

Another critical function of the liver is to maintain the plasma carbohydrate levels within a narrow, normal range \(^3,^4\). Excess glucose and fructose entering the blood after a meal are removed by hepatocytes, which convert the sugars to glycogen (glycogenesis) or other metabolites such as fatty acids and lactate. During fasting, the liver becomes an essential source of energy for other tissues by exporting glucose back into the blood. Initially, glucose is generated via depolymerization of the glycogen stored within hepatocytes (glycogenolysis). When hepatic glycogen reserves become exhausted, glucose is synthesized by hepatocytes from amino acids, lactate, pyruvate, and glycerol (gluconeogenesis).

**Xenobiotic biotransformation**

The liver is pivotal in the metabolism of most drugs and related xenobiotics. These compounds, often lipophilic, are rendered hydrophilic by hepatocytes via two phases of biotransformation \(^5\). In phase I, the compounds are converted to more polar metabolites for excretion by oxidation, reduction, or hydrolysis. Compounds that cannot be effectively excreted through phase I reactions are conjugated with an endogenous molecule such as glucuronic acid in a phase II reaction. Major enzymes involved in these reactions include the cytochrome P-450 superfamily that mediate phase I oxidation reactions, as well as uridine diphosphate (UDP)-glucoronyl transferases and glutathione S-transferases that catalyze phase II reactions.
**Lipid metabolism**

The liver plays a major role in lipid metabolism. Hepatocytes are capable of synthesizing cholesterol. The cholesterol is packaged into lipoproteins and secreted into the blood stream, or excreted via the biliary system. Additionally, the liver is the major site for synthesis of fatty acids, triglycerides, phospholipids, and lipoproteins. Triglycerides formed in the liver are either packaged into lipoproteins for export or metabolized within hepatocytes for energy production.

**Bile acid synthesis**

Hepatocytes are critical to the synthesis, secretion, and cycling of bile acids, and are the only cells that can enzymatically convert cholesterol to bile acids. The synthesis of bile acids helps to maintain cholesterol homeostasis, as it is a major route for eliminating cholesterol in the body. Another major function of bile acids is the emulsification, solubilization, and transport of dietary lipids, which enhances their absorption in the small intestine.

**Bilirubin removal**

The liver is responsible for disposing bilirubin, a toxic product derived from the breakdown of haem-containing compounds, predominantly haemoglobin from senescent erythrocytes. Bilirubin formed by phagocytic cells is released into the blood stream and transported by albumin. It is efficiently removed from the plasma by hepatocytes. In hepatocytes, the lipid-soluble bilirubin is converted to a water-soluble compound by conjugation with glucuronic acid. The conjugated bilirubin is secreted into the canaliculus as part of bile, and ultimately eliminated upon further reactions.
1.2 ADULT HEPATOCYTES

Although liver transplantation is the only effective treatment of end-stage liver diseases at present, severe donor shortage calls for the development of alternative treatment approaches. Due to the diverse and complex nature of liver functions, it has not been possible to support a failing liver with purely mechanical systems. Hepatocytes, the chief functional cells of the liver, have therefore become a prime component of cell therapy and tissue engineering approaches to liver repair and regeneration. These alternative strategies that are being developed include 1) hepatocyte transplantation; 2) implantable tissue constructs constituted by hepatocytes and their supportive scaffolds; 3) extracorporeal liver support systems.

In order to achieve clinical efficacy, all of these approaches require a sufficient mass of functional hepatocytes. This has proven to be a challenge as hepatocytes from adult livers are rather difficult to culture. Although hepatocytes can proliferate efficiently in vivo in response to liver damage, they possess low growth capacity in vitro. Furthermore, the large array of in vivo functions carried out by hepatocytes is mostly lost in culture. These issues limit the quantity and quality of hepatocytes available for treatment.

In the past several decades, the culture conditions of hepatocytes have been extensively studied in order to address these issues. These researches attempted to promote the proliferation of hepatocytes or to stabilize their differentiated phenotype by manipulating the soluble signals provided by the culture medium, homotypic (hepatocyte – hepatocyte) and heterotypic (hepatocyte – non-parenchymal cell) cell-cell interactions, and cell-matrix interactions. As a result, hepatocytes are able to undergo a few rounds of cell divisions under optimized conditions. However, such expansion efficiency is still not enough to meet the clinical requirement. Furthermore, even though hepatocyte functions can be better
maintained under certain conditions, a reciprocal control of growth and differentiation is often observed\textsuperscript{14,31,32}. To date, there has been very limited success in achieving both efficient hepatocyte expansion and long-term maintenance of hepatocyte functions. This has significantly impeded the progress of liver cell therapy and liver tissue engineering research.

1.3 LIVER STEM CELLS AND PROGENITORS

Due to the difficulty in culturing adult hepatocytes, researchers have looked to liver stem cells and progenitors as an alternative cell source. Compared with adult hepatocytes, these cells possess inherently high proliferation potential and are able to differentiate into functional hepatocytes under the right conditions. Furthermore, some of these cells are capable of differentiating into both hepatocytes and bile duct cells, thus raising the possibility of offering a wider range of liver functions\textsuperscript{33}.

Cells possessing progenitor qualities have been observed in adult liver. The most notable example is the oval cells residing in the periportal region of the liver\textsuperscript{34}. These cells feature ovoid nucleus, compact cell size, and high nucleus-to-cytoplasm ratio. It has been well documented that oval cells undergo rapid proliferation and infiltrate into the liver parenchyma when hepatocyte proliferation is inhibited during liver damage. It has also been suggested that oval cells are at least bipotential, capable of differentiation along either the hepatocyte or bile duct lineage. However, the isolation and culture methods of these cells are not well established at present. In addition, it has been reported that the adult liver contains the so-called “small hepatocytes”, which appear to be less differentiated and more proliferative than mature hepatocytes\textsuperscript{16,35}. 
Embryonic stem cells develop into hepatocytes during spontaneous differentiation. Recent studies have reported initial success in directing the differentiation process so that hepatocytes are preferentially derived. However, it remains a challenge to separate the resulting hepatocytic fractions from undifferentiated embryonic stem cells that may be tumorigenic in vivo.

Liver progenitors from extrahepatic sources have also been reported. Especially, stem cells derived from the bone marrow (BM), including hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), and the so-called MAPC (multipotent adult progenitor cells) have been described to “transdifferentiate” into hepatocytes both in animal models and in culture. However, at least with regard to the in vivo studies, it is still being debated whether the phenotypic change is due to true differentiation or the fusion of BM cells with hepatocytes.

Cells in the fetal liver lack many of the functions of mature hepatocytes. In fact, many of the important hepatocyte functions are acquired during perinatal stage or post birth (see section 1.4). On the other hand, cells from fetal livers posses much higher proliferation capacity than adult hepatocytes (section 1.4). Additionally, it has been shown in animal models that fetal liver cells readily repopulate diseased liver upon transplantation. Thus, fetal liver cells represent a population with liver progenitor qualities. Compared with some of the other liver progenitors, fetal liver cells are easy to isolate and have been cultured successfully for both basic science research and clinical studies (section 1.4). Furthermore, some adult liver progenitors, such as oval cells, are thought to resemble progenitors of the fetal liver in both phenotype and differentiation behavior. Therefore, fetal liver cells may provide a good model system for developing technologies relevant to the clinical applications of liver progenitors.
1.4 FETAL LIVER CELLS

1.4.1 Development of the liver

In mice, liver development initiates on embryonic day 8.5 – 9 (E8.5 – E9). In this initial stage, signals derived from the adjacent cardiac mesoderm, such as fibroblast growth factor 1 (FGF1) and FGF2, commit primitive pluripotent endodermal stem cells residing in the ventral wall of the foregut endoderm to form the liver diverticulum (the primary liver bud) 53-56.

Over the next 2 days, cells in the liver bud proliferate rapidly and migrate into the surrounding septum transversum 57. Signals from the mesenchyme of the septum transversum, such as bone morphogenetic proteins (BMPs), further induce the cells to form the definitive liver 58, 59. The embryonic liver at this stage consist of 1) pluripotent liver stem cells; and 2) their progeny, the transitory bipotential progenitors, hepatoblasts.

During E12 – E16, the fetal liver becomes the major site for embryonic hematopoiesis after invasion of the hematopoietic stem cells on E10.5 60, 61. Hepatic stem cells and progenitors, together with other stromal cells, foster the expansion of hematopoietic cells and the increase of their lineage diversity 62-65. On the other hand, hematopoietic cells produce oncostatin M (OSM) that induces the hepatic differentiation of hepatoblasts and their progeny 66.

In the late fetal and perinatal stages (E16 – E19 or E20 depending on the mouse strain), hematopoiesis in the liver diminishes as the hematopoietic cells migrate to spleen and bone marrow. Concomitantly, the liver undergoes a functional switch from a hematopoietic microenvironment to a metabolic organ, as many new genes related to postnatal liver functions are activated in fetal hepatocytes 67-69. The final
step of liver maturation in rodents takes place several days post birth (terminal differentiation). Throughout liver development, liver cells of various developmental stages proliferate continuously. Cell growth declines gradually as the liver matures, and is eventually arrested after birth.

Gene markers have been used to characterize the progression from pluripotent endodermal stem cells to mature hepatocytes. An early gene that signifies the differentiation of endodermal stem cells is α-fetoprotein (AFP), which is expressed in liver stem cells and hepatoblasts. Its expression diminishes as the cells develop into mature hepatocytes. Albumin (ALB), which belongs to the albuminoid gene superfamily as does AFP, is detectable in hepatoblasts. Its expression increases as hepatic differentiation progresses and reaches maximum in mature hepatocytes. Some researchers suggested that cytokeratin 19 (CK19), a protein marker of the bile duct cells in the adult liver, is also expressed by the bipotential hepatoblasts and its expression is lost as the cells differentiate along the hepatic lineage. In late gestational and perinatal stage, metabolic enzymes such as glucose-6-phosphatase (G6Pase) and tyrosine aminotransferase (TAT) begin to be expressed in perinatal hepatocytes as they prepare for their postnatal metabolic functions. Other hepatic enzymes, such as serine dehydratase (SDH) and tryptophan oxygenase (TO), are not induced until the terminal differentiation phase after birth. Several of the P-450 isoenzymes are also induced during this period.

### 1.4.2 Isolation of fetal liver epithelial cells and progenitors

Enrichment for fetal liver epithelial cells (will be called “fetal liver cells” henceforth) has been performed by depleting the hematopoietic cells of the fetal liver. Techniques such as panning, magnetic cell sorting (MACS), and FACS (fluorescence
activated cell sorter) sorting were applied to remove cells that express blood cell markers. The most commonly used hematopoietic markers were CD45 (leukocyte common antigen) and TER119 (an antibody recognizing the glycoprotein A expressed on all erythrocytes).

Several recent studies employed MACS and FACS sorting to purify postulated liver stem cells and hepatoblasts via positive selection. Combining FACS sorting and single-cell based assays, Suzuki et al. isolated prospective liver stem cells from E13.5 mouse fetal liver that express both CD49f and CD29 (α6 and β1 integrin subunits that bind to laminin), but not the hematopoietic markers CD45, TER119, and c-Kit. Anther cell membrane protein, the cell-cell adhesion glycoprotein E-cadherin, was used by Nitou and coworkers to positively select cells with hepatoblast characteristics from E12.5 mouse liver. Miyajima’s group reported that hepatoblasts could be isolated from E14.5 mouse livers based on their expression of delta-like leucine zipper kinase (Dlk, also known as Pref-1). Liv2, a cell surface molecule with unknown functions, was reported to be a specific marker for liver stem cells and progenitors from E9.5 to E12.5. These results were unified in a recent study performed by Shafritz’s group, which showed that AFP$^+/\text{ALB}^+$ cells in E12.5 mouse liver (about 2.5\% of total cells) possessed progenitor qualities such as the capacities for hepatic differentiation and liver repopulation. These cells were positive for E-cadherin, Liv2, and Dlk, and vice versa. On the other hand, the AFP$^+/\text{ALB}^+$ cells did not express hematopoietic markers CD45, TER119, and c-Kit. Only the expression of Sca-1 was shared by the hematopoietic and epithelial cell fractions.
1.5 SCOPE OF THESIS

As discussed earlier, the development of liver cell therapy and liver tissue engineering is hindered by the difficulty in culturing adult hepatocytes. Unlike hepatocytes, liver stem cells and progenitors possess high growth potential. *In vitro* studies and animal experiments have also demonstrated their ability to differentiate into functional hepatocytes and repopulate failing livers. Therefore, liver stem cells and progenitors represent a promising alternative cell source.

To generate an adequate mass of functional tissue from liver stem cells and progenitors for clinical applications, it is necessary to establish a culture system that supports their optimal proliferation and differentiation. To date, although culture methods have been developed for some liver progenitors, their effectiveness has not been characterized. Furthermore, due to the diversity of liver diseases and of the applied treatment regimes, cultured cells with specific qualifications (such as their developmental stage) may be required. At present, this ability has not been fully realized, as the impact of culture environment on liver stem cells and progenitors has not been fully explored.

In order to understand these issues, this thesis chose the fetal liver cells as a model. Compared with other liver stem cells and progenitors, fetal liver cells are relatively easy to isolate and culture, and readily differentiate into hepatocytes both *in vitro* and *in vivo*. Consequently, the design, execution, and interpretation of experiments would be more straightforward.

Although various methods have been developed to purify liver stem cells and progenitors from the fetal liver (see section 1.4.2), it was decided that bulk fetal liver cells, depleted of non-adherent hematopoietic cells, would be used in this thesis. This decision was based on several considerations. First, sophisticated cell purification procedure may be too time-consuming and therefore not practical for clinical
applications. Second, preliminary experiments showed that TER119+/CD45+ cells account for about 65% of the total cells, and TER119+ cells constitute about 90% of the TER119+/CD45+ populations (data not shown). The TER119+ cells can be easily depleted by red blood lysis. Furthermore, the unlysed erythrocytes and non-adherent TER119−CD45+ cells in the culture can be effectively removed by changing the culture medium. Therefore, the epithelial cell fraction can be significantly enriched by these simple techniques. Third, even with a pure liver stem cell starting population, the resulting culture is likely to be a mixture of cell populations at different developmental stages and even of different lineages due to simultaneous cell proliferation and differentiation. Last, it is anticipated that an optimal culture system will selectively promote the propagation and differentiation of liver progenitors and their progeny.

This thesis attempted to explore the influence of culture parameters on growth and differentiation of fetal liver cells. Specifically, signals provided by soluble factors and culture substrates were evaluated. The investigation of soluble factors is described in Chapter 2. The study was designed to examine the effectiveness of currently used culture medium formulations, to characterize the effect of various soluble factors on the growth and differentiation dynamics of fetal liver cells, and to develop an optimal medium formulation based on these findings. Results from this study would be applicable to all three categories of liver cell therapy and liver tissue engineering, namely cell transplantation, implantable cell/scaffold composites, and extracorporeal liver support systems. To date, the scaffold design requirement for fetal liver cell culture has not been defined. Therefore, Chapter 3 and Chapter 4 focused on the three-dimensional culture of fetal liver cells under the medium condition developed in Chapter 2. In Chapter 3, representative 3D scaffolds currently used in liver tissue engineering were assessed regarding their effectiveness in supporting fetal liver cell cultures. This study was intended as a general survey in
order to obtain information on scaffold properties that are possibly crucial to the
development of fetal liver cells. Based on results from Chapter 3, a more detailed
study was performed in Chapter 4 to investigate the impact of scaffold properties, in
particular the bulk properties such as degradation behavior and mechanical
characteristics. Findings from Chapter 3 and 4 would be relevant to the design of
implantable or extracorporal devices, but may also provide insights on how to improve
and/or control the cell behavior for cell transplantation purpose.

It is expected that findings from this thesis would help to improve the current
culture technologies for fetal liver cells in that cell expansion and maturation can not
only be maximized but can also be controlled. Furthermore, it is anticipated that these
findings could be extended to the applications of other liver stem cells and
progenitors.
BIBLIOGRAPHY


CHAPTER 2
MEDIUM OPTIMIZATION FOR FETAL LIVER CELL CULTURE

2.1 INTRODUCTION

In order to generate a large number of functional hepatocytes from fetal liver cells, it is necessary to establish a culture system that supports their optimal proliferation and maturation. The methods established for fetal liver cells would also provide a foundation for developing culture techniques for other liver progenitors.

Some of the factors that can influence adult hepatocytes also exert regulatory effects on fetal liver cells. For example, hepatocyte growth factor (HGF), whose combination with epidermal growth factor (EGF) has the most mitogenic effect on adult hepatocytes \(^1\), was found to be critical for the proliferation of hepatic stem cells and hepatoblasts \(^2\)-\(^4\). Dexamethasone, a synthetic glucocorticoid, is a key culture component that improves the viability of mature hepatocytes and preserves their functions \(^1\), \(^5\). In the fetal liver, dexamethasone suppresses the expression of \(\alpha\)-fetoprotein (AFP) and up-regulates albumin synthesis \(^6\). In culture, it induces E12.5 fetal liver cells to acquire characteristics of perinatal hepatocytes, as demonstrated by the expression of glucose-6-phosphatase (G6Pase) and tyrosine amino transferase (TAT) \(^7\).

Other molecules have been found to play roles in fetal liver development. Most notably, oncostatin M (OSM), a member of the interleukin-6 (IL-6) family, was shown to be a potent stimulator of the maturation of fetal liver cells along the hepatocytic lineage \(^8\), \(^9\). Dexamethasone is absolutely required for the actions of OSM, and augments its effects in a dose-dependent fashion, in the range of 0 to 1 \(\mu\)M \(^8\). The maturation of fetal liver cells induced by OSM is further enhanced by the
presence of dimethylsulfoxide (DMSO) and nicotinamide, two pivotal factors that extend the survival, DNA synthesis, and functional preservation of adult hepatocytes in culture.

Fetal liver cell culture media reported in previous studies took advantage of these findings as well as the existing technology developed for adult hepatocyte cultures. Basal media that have been used ranged from standard formulations such as DMEM and DMEM/F12 to those specifically designed for long-term culture of adult hepatocytes, such as Williams’ E medium and the chemically defined Block’s medium. These basal media were supplemented with various components that are commonly required for the well-being of many cell types, including serum or bovine albumin, L-glutamine, non-essential amino acids, and insulin. In addition, compounds that regulate fetal liver cells, such as dexamethasone and nicotinamide, were often supplied. Finally, depending on the purpose of the experiment, factors that regulate the growth or differentiation of fetal liver cells were applied. Typically, EGF and HGF were added to stimulate growth, whereas OSM was used to induce maturation.

Some researchers adopted a two-step approach in attempts to achieve both maximal proliferation and maturation. In this case, the fetal liver cells first underwent expansion in a growth-stimulating medium. Subsequently, they were induced to differentiate under a second condition that encouraged maturation and often reciprocally repressed growth. In other studies, a single medium combining the mitogens and differentiation factors were used for the whole culture period.

Although a wide variety of fetal liver cell culture media have been described, there has been no direct comparison of their effectiveness. Nor has the kinetics of cell growth and maturation been delineated under complex culture conditions that combine growth- and differentiation-stimulating factors. Detailed studies that address these
issues will not only help to improve the current culture methods, but also provide insights on how to control the growth and differentiation of fetal liver cells.

In this study, three representative medium conditions were directly compared. The first medium, containing HGF and EGF as the key regulatory factors, has been used to support the clonal expansion of liver stem cells isolated from E13.5 mouse livers. The second formulation was designed to induce the morphological and functional maturation of E14.5 fetal liver cells based on the use of OSM, DMSO, high concentration of dexamethasone, and the basal medium Williams’ E. The third condition, a two-step approach, combined the first two media in a sequential manner to stimulate both growth and differentiation. Based on these results, the medium formulation was further modified to achieve both optimal growth and differentiation. The underlying mechanisms of the growth and differentiation responses to the tested conditions were also discussed.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Mice used in this study were from The Jackson Laboratory (Bar Harbor, Maine). Trypsin stock solution, calcium/magnesium-free Dulbecco’s phosphate-buffered saline (DPBS), antibiotic-antimycotic, L-glutamine, non-essential amino acids, all basal culture media, human EGF, Trypsin/EDTA, as well as fetal bovine serum (FBS) used in cell trypsinization and MTS assays were purchased from Invitrogen Gibco (Grand Island, NY). FBS used for fetal liver cell processing and cultures, as well as the HEPES buffer solution, were purchased from Atlanta Biologicals (Lawrenceville, GA). Dexamethasone, nicotinamide, ascorbic acid
diphosphate, DMSO, and insulin solution were acquired from Sigma (St. Louis, MO). Human HGF and mouse OSM were purchased from R&D Systems (Minneapolis, MN). Pharm Lyse solution was from BD Pharmingen (San Jose, CA). Collagen stock solution was purchased from Angiotech BioMaterials (Palo Alto, CA). Tissue-culture treated polystyrene plates, cell strainers, and collagen-coated chamber slides were obtained from BD Discovery Labware (San Jose, CA). Beta-mercaptoethanol, Tris-HCl, EDTA, and Triton-X100 stock solutions were from American Bioanalytical (Natick, MA). Paraformaldehyde stock solution was acquired from Electron Microscopy Sciences (Hatfield, PA). The MTS assay was performed using a commercially available kit from Promega (Madison, WI). PicoGreen dsDNA Quantitation kit was acquired from Molecular Probes (Eugene, OR). Albumin ELISA kit was purchased from Bethyl Laboratories (Montgomery, TX).

2.2.2 Isolation and processing of fetal liver cells

Methods for the isolation and processing of fetal liver cells were modified from previously reported protocols \(^2,^{10}\). Timed pregnancies were set up using C57Bl/6 mice. Noon of the day a vaginal plug was found was considered as 0.5 embryonic day (E0.5). On E14.5, fetuses were retrieved from the pregnant mice. Each pregnant mouse yielded 5 – 12 fetuses. Fetal livers were aseptically dissected from the fetuses under dissection microscope and maintained in DPBS + 1% antibiotic-antimycotic on ice until use. To extract fetal liver cells, fetal livers were treated with 0.1% trypsin for 10 minutes at 37°C. Subsequently, the livers were triturated with P1000 pipettes to achieve a single cell suspension, and 10% FBS-containing medium was added to neutralize the trypsin. The cells were pelleted and resuspended in Pharm Lyse solution to lyse erythrocytes. After 10 minutes of incubation in the dark at room temperature (RT), 10% FBS-containing medium was added to dilute the Pharm Lyse.
The cells were pelleted at 1,100 rpm for 5 minutes, resuspended in the same medium, and re-pelleted at 1,100 rpm for 5 minutes. The cell pellet was resuspended in 1 ml of culture medium, and filtered with a cell strainer (40 µm pore size). Another ml of medium was used to rinse the filter and combined with the cell suspension. In average, this process yields 1.07X10^8 fetal liver cells from each pregnant mouse (n = 11).

2.2.3 Culture of fetal liver cells

The compositions of the culture media studied in this chapter are described in Table 2.1. Incomplete culture media, which were used for the first day of culture, refer to media that were not supplemented with insulin, EGF, HGF, OSM, or DMSO. Complete culture media included all those factors and were used for the rest of the culture period.

On day 0 (day of culture set-up), collagen working solution was prepared by diluting the stock (2.9 mg/ml) 1:10 with sterile water. Twelve-well tissue-culture treated polystyrene plates were coated with the collagen working solution at 37°C for 1 hour, and rinsed with sterile water. The wells were then stored in DPBS until use.

The processed fetal liver cells were counted by Trypan Blue exclusion, and diluted with the incomplete culture medium of interest to 2X10^5 cells/ml. This suspension was plated onto the collagen-coated plates at 1 ml/well, resulting in a plating density of 5X10^4 cells/cm^2. After 20 – 24 hours of culture, culture supernatant was removed and the culture wells were rinsed with DPBS to remove non-adherent cells. Complete culture medium was then added at 1 ml/well. Culture medium was changed every 2 – 3 days thereafter.
<table>
<thead>
<tr>
<th>Table 2.1 Formulations of the culture media studied in this chapter.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Basal medium</td>
</tr>
<tr>
<td>FBS</td>
</tr>
<tr>
<td>L-glutamine</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>Antibiotic-antimycotic&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>HEPES</td>
</tr>
<tr>
<td>Dexamethasone</td>
</tr>
<tr>
<td>Nicotinamide</td>
</tr>
<tr>
<td>Ascorbic acid diphosphate</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Insulin</td>
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<tr>
<td>EGF</td>
</tr>
<tr>
<td>HGF</td>
</tr>
<tr>
<td>OSM</td>
</tr>
<tr>
<td>DMSO</td>
</tr>
</tbody>
</table>

<sup>1</sup> Contains 10,000 U/ml of penicillin (base), 10,000 µg/ml of streptomycin (base), and 25 µg/ml of amphotericin B in 0.85% saline (information provided by the product manufacturer).
2.2.4 Assessment of cell growth

Cell growth was monitored by MTS assay and DNA assay. The MTS assay measures the conversion of a tetrazolium compound into aqueous, soluble formazan by dehydrogenase enzymes found in metabolically active cells. In addition, the total amount of DNA extracted from each culture well was quantified with the PicoGreen dsDNA Quantitation kit.

The culture medium used for the MTS assay consisted of phenol-red-free DMEM/F12 + 10% FBS + 1% L-glutamine + 1% non-essential amino acids + 1% antibiotic-antimycotic. To perform the MTS assay, culture media were removed and the wells were rinsed with DPBS to remove residual media with phenol red. Subsequently, 1 ml of MTS medium was added to each well. This was followed by the addition of 200 µl/well of PMS/MTS mixture, which was pre-mixed and frozen in aliquots at –20°C according to the manufacturer’s instructions. The plate was placed in a cell culture incubator for 2 hours. Afterwards, 120 µl of suspension (in triplicate) from each culture well was transferred to a 96-well plate. Optical absorbance was measured in dual mode (at 490 nm with a reference at 630 nm) with an ELx808 microplate reader (BIO-TEK Instruments, Winooski, VT). On day 0, MTS assay was performed on a serial titration of the processed fetal liver cells (8X10^6 – 7.8X10^3 cells/well at 1:2 titration) to test the linearity of the assay.

Following the MTS assay, the supernatant was aspirated from the culture wells, which were then rinsed with DPBS. Subsequently, the cells were detached by incubation with Trypsin/EDTA at 37°C for 10 minutes. The cells were pelleted, resuspended in 200 µl of DPBS, and stored at –80°C until use. To extract DNA, these samples were thawed, brought to RT, and mixed with 200 – 400 µl of lysis solution (10 mM Tris-HCl + 1 mM EDTA + 0.2% Triton-X100, pH 7.5). The cell suspension was sonicated on ice for 1 minute using a sonicator (VibraCell model, Sonics &
Materials, Newton, CT) with an output level of 4 and a 40% duty cycle. The sonicated samples were stored at –80ºC until use. On the day of DNA analysis, the sonicated samples were thawed and quantified for DNA according to the PicoGreen kit manufacturer’s instruction. Fluorescence was measured with a CytoFluor 2350 fluorimeter (Millipore, Billerica, MA). Standard curves using serially diluted day 0 fetal liver cells (8X10^6 – 7.8X10^3 cells at 1:2 titration) were used to test the linear ranges of the assay at various sample dilutions.

2.2.5 Analysis of hepatocyte functions

Albumin synthesis and glycogen accumulation were analyzed to evaluate the development of hepatocyte functions in the fetal liver cell cultures.

At each medium change, spent culture medium from each well was collected and centrifuged to pellet non-adherent cells and debris. The top 80-90% of the spun medium was collected and stored at –80ºC until analysis. Mouse albumin secreted into the culture media was measured by ELISA assay according to the kit manufacturer’s instructions. Optical absorbance was measured at 490 nm on the same plate reader as the MTS assay. The culture media alone did not give any positive reading, indicating that cross-reactions between the ELISA antibody and the FBS contained in the media were negligible.

Periodic acid-Schiff (PAS) staining of the cultures was performed to assess glycogen accumulation. Cultures for PAS staining were set up in collagen-coated chamber slides in parallel with those in 12-well plates on day 0. The number of cells seeded into the chamber wells was scaled according to available culture surface to maintain the plating density at 5X10^4 cells/cm^2. At designated time points, spent culture media was aspirated. The chamber wells were quickly rinsed with DPBS, and fixed with 4% paraformaldehyde at RT for 10 minutes. The fixed wells were rinsed
twice with DPBS, at RT for 5 minutes each. The rinsed wells were air dried for at least 1 hour before being sealed and stored at –80ºC. For staining, the chamber wells were brought to RT, air dried, and submitted to Research Histology at Yale University for PAS staining.

2.2.6 Statistics

All experiments were performed at least 3 times using cells from different animals, with each condition tested in 2 - 6 replicates. Similar trends were observed in all experiments. Student’s $t$-test was used to evaluate the statistical significance where indicated. Significance level was set at $p < 0.05$.

2.3 RESULTS

2.3.1 Fetal liver cells cultured in media E and WD

This set of experiments compared the culture of E14.5 mouse fetal liver cells in two representative medium formulations based on HGF/EGF and OSM/DMSO as the key regulating factors, respectively, as well as a two-step approach in which the two media were applied sequentially. The fetal liver cells were cultured: 1) for 2 weeks in the HGF/EGF-containing medium (medium E); 2) for 2 weeks in the OSM/DMSO based medium (medium WD); 3) in medium E for 1 week followed by another week in medium WD (medium EWD).

Morphological changes

After the removal of non-adherent cells (predominantly blood cells) at day 1, only about 5% of the seeded cells remained in the culture wells. The attached cells
included mostly nonparenchymal cells and a few clusters of cells with very small cell size (5 – 10 µm). In medium E and medium WD, these small cells increased in number over time, and became drastically different in morphology starting around day 4 - 5.

As reported, medium WD induced a cell morphology resembling that of more differentiated hepatocytes. Starting around day 3 - 4, the small cells in the culture gradually enlarged in size, reaching 20 –40 µm at day 15, and exhibited the polyhedral shape that is typical of differentiated hepatocytes. These cells also possessed spherical nuclei with one or more prominent nucleoli, another signature of hepatocytes. Over time, a portion of the cells became binucleate, similar to what is observed in adult liver (Figure 2.1). Compared with the WD cultures, the small cells in medium E appeared to proliferate much faster. However, throughout the 2-week culture period, they remained as tightly packed, round, and small cells, often with high nucleus to cytoplasm ratio. Few differentiated hepatocytes were observed. When the culture medium was switched to WD (EWD), cells originally cultured in medium E started to display hepatocyte morphology within 2 days: they increased in cell size, became polygonal, and the number of binucleate cells increased over time.

**Cell growth**

Consistent with visual inspection, results from DNA assay and MTS assay indicated that cells cultured in medium E initially underwent rapid expansion (Figure 2.2). At day 7, total cellular DNA increased by 219.3 fold compared with day 1. However, the growth declined after this period, as reflected by a much slower increase in the total DNA and a decrease in the MTS activity after 1 week. On the other hand, the DNA content and MTS activity of cells cultured in medium WD increased slowly
Figure 2.1  Morphology of E14.5 mouse fetal liver cells cultured for 2 weeks in medium WD; for 2 weeks in medium E; or for 1 week in medium E followed by another week in medium WD (EWD). Magnification = 40X.
Figure 2.2  Proliferation of E14.5 mouse fetal liver cells as measured by DNA assay (A) and MTS assay (B) when cultured in medium E, WD, and EWD.
but steadily over time, and eventually caught up with those in medium E. By switching the medium from E to WD at day 7, the decline of cell growth observed in E cultures was somewhat ameliorated: the total DNA increased at a higher rate, reaching a level 20% and 37% higher than those in medium E and WD, respectively, at the end of the culture. However, the switch did not restore the MTS activity: it decreased during the first 2 days of the switch before recovering to a level similar to those in medium E and WD.

**Albumin synthesis**

The level of albumin synthesis in medium E was much higher than that in medium WD for a large part of the experiment (the first 10 – 11 days, Figure 2.3). The total secretion rate and specific secretion rate in medium E climbed rapidly during the first week, reaching to 8.4 and 6.4 fold of those in medium WD, respectively, on day 8. However, the synthesis decreased steadily thereafter, eventually to a level similar to that in medium WD around day 12. When switching from medium E to medium WD at day 7, albumin secretion dropped even more significantly and only recovered to the level of media E and WD at the end of the experiment.

**Glycogen accumulation**

The ability of the cultured cells to produce and store glycogen was also examined under the three conditions. After one week of culture, many cells in medium WD showed an intense staining that indicates high level of glycogen accumulation (Figure 2.4). All of these stained cells had the appearance of more differentiated hepatocytes. The staining pattern of the WD cultures remained similar till the end of the 2-week culture. In contrast, only a small percentage of the cells in medium E were stained after 1 week. The stained cells also displayed the
Figure 2.3 Albumin secretion of E14.5 mouse fetal liver cells cultured in medium E, WD, and EWD, as indicated by the total secretion rate (µg albumin/well-day, A) and the specific secretion rate (µg albumin/µg DNA-day, B).
Figure 2.4  Glycogenesis of E14.5 mouse fetal liver cells cultured in medium WD for 1 week (A) or 2 weeks (B), in medium E for 1 week (D) or 2 weeks (E), and in EWD for 2 weeks (F). The stained cells in all three cultures displayed the morphology of mature hepatocytes (C). Magnification = 4X (A - B, D - F) or 40X (C). Scale bar = 500 µm (A - B, D - F) or 100 µm (C).
differentiated hepatocyte morphology. However, only a very low level of staining remained in the E cultures after 2 weeks. By switching the medium from E to WD at day 7, many intensely stained cells were observed at day 15, all of which exhibited the typical hepatocyte morphology.

2.3.2 A Modified medium condition for fetal liver cell culture

Based on the results in section 2.3.1, experiments in this section investigated whether the growth and differentiation of fetal liver cells could be further improved by modulating the regulatory factors and the timing of their addition. E14.5 mouse fetal liver cells were cultured: 1) for 2 weeks in medium E; 2) for 2 weeks in a modified medium supplemented with HGF, EGF, OSM, and DMSO (medium DD); 3) in medium E for 1 week followed by another week in medium DD.

Morphological changes

The morphology of the E cultures was similar to what was described in section 2.3.1 (Figure 2.5). In DD medium, clusters of small hepatocytes started to emerge around day 4 – 5. Similar to the WD cultures, these cells enlarged in size and acquired the morphology of mature hepatocytes over time. Yet unlike those in medium WD, cells cultured in medium DD demonstrated a higher expansion capacity. Similar to the EWD cultures, cells originally cultured in medium E started to display hepatocyte morphology within 2 days of switching to medium DD.

Cell growth

Similar to what was previously observed, cell proliferation in medium E was rapid initially but stalled after one week, as indicated by a plateau in the DNA content after day 5 and a decline in MTS activity after day 7 (Figure 2.6). In contrast, the
Figure 2.5  Morphology of E14.5 mouse fetal liver cells cultured for 2 weeks in medium DD, for 2 weeks in medium E, or for 1 week in medium E followed by another week in medium DD (EDD). Magnification = 40X.
Figure 2.6  Proliferation of E14.5 mouse fetal liver cells as measured by DNA assay (A) and MTS assay (B) when cultured in medium E, DD, and EDD.
DNA content and MTS activity of the DD cultures increased at a rate similar to those in medium E during the first 5 days but continued to rise thereafter, albeit at a slower rate. Similar to the switch from medium E to medium WD, changing the medium from E to DD at day 7 prolonged the increase of DNA content. Unlike the EWD condition, this switch did not cause a drop in MTS activity and restored it to a level significantly higher than that of the E cultures ($p < 0.05$ for day 11, 13, 15). The DD and EDD conditions both led to significantly higher cell expansion (measured by the increase in total cellular DNA) compared with medium E: at the end of a 15-day culture, cell expansion in DD and EDD cultures were 2.3 and 1.6 fold higher than that in medium E, respectively ($p < 0.05$).

**Albumin synthesis**

Albumin synthesis in medium E followed a trend similar to what was described in 2.3.1: it increased rapidly during the first week but declined thereafter (Figure 2.7). In contrast to the EWD condition, switching the E cultures to medium DD at day 7, when albumin secretion was at its peak, stabilized the secretion at this level. Interestingly, although albumin secretion in medium DD was low initially, it increased with time in an almost linear fashion and eventually surpassed the secretion level in EDD. At the end of a 15-day culture, the total secretion rate and specific secretion rate in medium DD were 1.9 and 1.6 times higher than those in EDD, respectively ($p < 0.05$).

**Glycogen accumulation**

Glycogenesis of the three cultures is demonstrated in Figure 2.8. As described before, the cultures in medium E contained only a few intensely stained hepatocytes at day 7 and the staining was largely diminished at day 15. This was overcome by
Figure 2.7  Albumin secretion of E14.5 mouse fetal liver cells cultured in medium E, DD, and EDD, as indicated by the total secretion rate (µg albumin/well-day, A) and the specific secretion rate (µg albumin/µg DNA-day, B).
Figure 2.8  Glycogenesis of E14.5 mouse fetal liver cells cultured in medium DD for 1 week (A) or 2 weeks (B), in medium E for 1 week (C) or 2 weeks (D), and in EDD for 2 weeks (E). After 2 weeks of culture in medium DD or EDD, both intensely stained mature hepatocytes (F) and intermediately stained small cells (G) were observed. Magnification = 4X (A - E) or 40X (F - G). Scale bar = 500 µm (A – E) or 100 µm (F – G).

---

2 Figure 2.8C is the same as Figure 2.4D; Figure 2.8D is the same as Figure 2.4E.
switching the medium to DD at day 7: a large number of intensely stained hepatocytes were observed at day 15. The staining of DD cultures was unremarkable at day 7. However, by day 15, the staining level in DD was the highest among all culture conditions (including WD and EWD). Interestingly, the staining was contributed by two groups of cells with distinct morphologies: large hepatocytes with intense staining, and intermediately stained clusters of tightly packed small cells that did not resemble differentiated hepatocytes. The latter was also observed in the EDD cultures, but the number of clusters was lower than in DD.

**Overall comparison**

When all the culture conditions were compared together, cell growth and albumin secretion appeared to be the highest in medium DD. In average, cell expansion (measured by the fold of increase in cellular DNA) in medium DD was about 2 fold of that under other conditions. At the end of a 2-week culture, the total albumin secretion rate and the specific albumin secretion rate in medium DD were 2 - 4 and 2 - 3 times of the maximal values under other conditions, respectively.

### 2.4 DISCUSSION

#### 2.4.1 Rationale and summary of results

The purpose of this study was to establish a medium condition that promotes optimal growth and hepatic differentiation of mouse fetal liver cells. The study began with a comparison of media E, WD, and EWD, respectively representing published methods that support growth, hepatocyte differentiation, or both growth and differentiation (results summarized in Table 2.2). The outcome of the experiments
was unexpected. Rapid cell proliferation and albumin synthesis were promoted in medium E but could not be sustained beyond one week of culture (Figure 2.2 and Figure 2.3). Furthermore, albumin secretion in medium WD was much lower than in medium E for most of the culture period (Figure 2.3), suggesting that the WD cultures either synthesized less albumin on a per-cell base or contained a lower percentage of albumin-secreting cells. This was especially surprising because only a few cells in medium E possessed the mature hepatocyte morphology and glycogen storage ability that were prevalent in cultures maintained in medium WD (Figure 2.1 and Figure 2.4).

Finally, compared with E and WD, the two-step approach (EWD) prolonged the increase of cellular DNA (Figure 2.2), induced morphological maturation (Figure 2.1) and glycogenesis (Figure 2.4), but could not restore albumin secretion (Figure 2.3). These results suggested that, with regard to their intended usage, the tested methods are not as effective as previously reported \(^2,^3,^10\).

Table 2.2 Summary of the comparison of media E, WD, and EWD.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell Growth</th>
<th>Morphological Maturation</th>
<th>Albumin Synthesis</th>
<th>Glycogen Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WD</td>
<td>Slow</td>
<td>Yes</td>
<td>Low</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Large hepatocytes)</td>
</tr>
<tr>
<td>E</td>
<td>Wk 1: high(^3) Wk 2: declined</td>
<td>No</td>
<td>Wk 1: high Wk 2: declined</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Large hepatocytes)</td>
</tr>
<tr>
<td>EWD</td>
<td>Prolonged</td>
<td>Yes</td>
<td>Not restored</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Large hepatocytes)</td>
</tr>
</tbody>
</table>

\(^3\) “Week” is abbreviated as “Wk”.


Since the outcome of the above experiment was less than satisfying, the second part of the study investigated whether the growth and differentiation of fetal liver cells could be enhanced by modulating the regulatory factors and the timing of their supplementation (results summarized in Table 2.3). A modified medium, DD, was designed based on E and WD to contain both growth- and differentiation-stimulating factors. Replacing WD with this medium in the two-step approach stabilized albumin secretion at the peak level achieved in medium E (Figure 2.7). When used alone, medium DD led to the highest cell expansion among all conditions and was able to sustain the growth for 2 weeks (Figure 2.6). Hepatic differentiation, demonstrated by morphological maturation (Figure 2.5) and glycogenesis (Figure 2.8), was also readily induced. Furthermore, albumin secretion in medium DD increased continuously over time, and became significantly higher than in all other cultures (Figure 2.7). These results are consistent with previous findings that the combination of HGF and OSM enhances the growth and hepatic differentiation of putative hepatic progenitors.\(^4\)

Table 2.3 Summary of the comparison of media E, DD, and EDD.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell Growth</th>
<th>Morphological Maturation</th>
<th>Albumin Synthesis</th>
<th>Glycogen Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>Wk 1: high</td>
<td>No</td>
<td>Wk 1: high</td>
<td>+/- (Large hep)(^4)</td>
</tr>
<tr>
<td></td>
<td>Wk 2: declined</td>
<td></td>
<td>Wk 2: declined</td>
<td></td>
</tr>
<tr>
<td>EDD</td>
<td>Prolonged</td>
<td>Yes</td>
<td>Stabilized at wk 1 level</td>
<td>+++ (Large/small hep)</td>
</tr>
<tr>
<td>DD</td>
<td>Highest</td>
<td>Yes</td>
<td>Highest</td>
<td>++++ (Large/small hep)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Continued increase</td>
<td></td>
</tr>
</tbody>
</table>

\(^4\) “Hepatocytes” is abbreviated as “hep”.
2.4.2 Effects of regulatory signals and timing of their addition

Suzuki et al. previously reported that HGF plays multiple roles in the development of liver stem cells isolated from E13.5 livers (Figure 2.9)\(^9\). First, HGF directly mediates the early transition of albumin-negative (ALB\(^{-}\)) stem cells to bipotential, transitory albumin-positive (ALB\(^{+}\)) hepatic precursors that resemble hepatoblasts. Second, it encourages the bipotential ALB\(^{+}\) precursors to become committed ALB\(^{+}\) hepatocyte precursors. Last, HGF functions as a potent mitogen for both ALB\(^{-}\) stem cells and differentiating ALB\(^{+}\) precursors. On the other hand, OSM inhibits the proliferation of ALB\(^{-}\) stem cells and has no mitogenic effect on ALB\(^{+}\) precursors. However, OSM, but not HGF, induces the ALB\(^{+}\) precursors to further differentiate into mature hepatocytes.

These findings could help to understand the complex growth and differentiation dynamics observed in this study. The fetal liver cells used for this study were not further fractioned, and thus contained both ALB\(^{-}\) stem cells and ALB\(^{+}\) precursors. In medium E, HGF converted the ALB\(^{-}\) stem cells to ALB\(^{+}\) precursors and strongly stimulated their proliferation, resulting in the initial rapid increase in DNA content, MTS activity, and albumin secretion. However, the maturation process was halted in the absence of OSM. This was demonstrated by the lack of morphological maturation, as well as a very low level of glycogenesis that was likely to be induced by dexamethasone\(^8\). In medium WD, OSM efficiently induced the maturation of the ALB\(^{+}\) hepatocyte precursors existing in the starting population, giving rise to glycogenic cells with mature hepatocyte morphology. Yet, in absence of HGF, the number of these cells increased slowly due to low mitogenic stimulation and a lack of new ALB\(^{+}\) precursors generated from ALB\(^{-}\) stem cells. Hence, the PAS staining pattern remained similar over time. The albumin secretion rates, which evaluate the average secretion level of the whole culture, also remained low.
Figure 2.9. Proposed mechanism of hepatic stem cell proliferation and differentiation regulated by HGF and OSM. Black horizontal arrows indicate the direction of differentiation, while black arc-shaped arrows symbolize self-renewal or proliferation. Red and blue arrows represent up-regulation and down-regulation, respectively. The arrow size illustrates the magnitude of the regulatory effect.
Medium DD is essentially medium E supplemented with ascorbic acid, OSM, DMSO, and more dexamethasone, all of which are present at the same concentrations in medium WD as well (Table 2.1). Therefore, it is not surprising that both of the two-step approaches, EWD and EDD, induced maturation of the ALB$^+$ precursors generated and expanded in medium E. However, switching from medium E to WD caused noticeable setbacks in MTS activity and albumin secretion, which were not observed with EDD. One possible reason could be that E and DD share the same basal medium DMEM/F12, the composition of which differs from the Williams’ E that WD is based on. Consequently, an adaptation phase was required with the E-to-WD switch but not with EDD. This hypothesis is supported by a previous study on fetal liver cell culture, in which switching from αMEM to Williams’ E medium either led to reduced cell number or could not restore albumin secretion$^{16}$. Therefore, two-step approaches combining basal media with rather different compositions may not be optimal. Second, HGF contained in medium DD stimulated the continual proliferation and differentiation of ALB$^+$ precursors, and thus helped to stabilize the levels of albumin secretion and MTS activity. The effect of HGF was further reflected in the PAS staining pattern of EDD cultures: besides the intensely stained mature hepatocytes that were also observed in WD and EWD, there existed intermediately stained small cells that were possibly newly generated, not fully differentiated hepatocytes. Therefore, the presence of HGF even in the so-called “maturation phase” could be beneficial.

When medium DD was used alone, the growth and maturation stimulators in the medium formed an “assembling line”: committed hepatic precursors were constantly generated owing to HGF, which induced the proliferation and early differentiation of ALB$^-$ stem cells and ALB$^+$ precursors. Subsequently, the hepatic precursors matured into hepatocytes under the stimulation of OSM. The concerted
actions of HGF and OSM resulted in an apparent association of growth and
differentiation, as reflected by the simultaneous increases in DNA content and
albumin secretion throughout the culture period. Additionally, similar to EDD, both
intensely stained hepatocytes and intermediately stained small cells were observed in
PAS staining.

Compared with EDD, cell growth, albumin synthesis, and glycogenesis were
further enhanced when medium DD was used throughout the culture period. The
improvements may be due to the presence of ascorbic acid, which is known to protect
both adult and fetal hepatocytes against apoptosis\textsuperscript{20, 21}. It is possible that, in medium
E, the ALB\textsuperscript{−} stem cells and ALB\textsuperscript{+} precursors were lost over time in the absence of the
protective agents, as demonstrated by the decline of cell growth and albumin secretion
after their initial ascent. Hence, EDD cultures were likely to contain less stem cells
and precursors than cultures maintained in medium DD alone, in which the cells were
protected from the very beginning. Consequently, after the medium switch, albumin
secretion increased at a much slower rate in EDD than in DD. This is further
illustrated by the pattern of PAS staining, wherein more extensive staining and more
intermediately stained small cells were observed in DD than in EDD. Therefore,
supplying protective reagents from the very beginning could augment cell growth and
hepatic differentiation.

In summary, results from this study show that the growth and differentiation
kinetics of fetal liver cells are influenced not only by the regulatory factors that are
present, but also by the timing of their addition. HGF is required throughout the
culture to induce the differentiation of ALB\textsuperscript{−} stem cells into hepatic precursors and to
ensure the continual proliferation of stem cells and precursors. The constant presence
of protective agents such as ascorbic acid helps to enhance cell survival, resulting in
improved cell expansion and hepatocyte functions. Finally, timing of the addition of
maturation factors such as OSM determines the kinetics of cell differentiation. In the presence of HGF, addition of OSM from the very beginning results in concurrent growth and differentiation.
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CHAPTER 3
FETAL LIVER CELL CULTURE ON THREE-DIMENSIONAL SCAFFOLDS

3.1 INTRODUCTION

Three-dimensional (3D) scaffolds play a central role in liver tissue engineering. Compared with two-dimensional (2D) culture surfaces, these scaffolds have a large surface area-to-volume ratio that potentially allows the growth of a large cell mass that is required for clinical efficacy. They also provide a 3D space for potential tissue formation and offer temporary mechanical support during this process. Furthermore, the scaffold serves as a delivery system that enables the transplantation of organized cells/tissues rather than single cells.

Scaffolds constructed from both synthetic and natural materials have been studied for liver tissue engineering. Among the synthetic materials, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer poly(lactic-co-glycolic acid) (PLGA) have been studied extensively. These polymers are biodegradable, biocompatible, and have been approved by the Food and Drug Administration (FDA) for applications such as wound dressings and surgical sutures. The polymers are fabricated into highly porous structures that can house a large number of hepatocytes, facilitate the transport of oxygen, nutrients and metabolic wastes, and allow vascular in-growth. Concomitant with the increase in cell mass, the scaffold degrades via hydrolytic cleavage of the ester bonds, leaving only the naturally generated tissue consisting of the transplanted cells and the in-grown host tissues.

Alternatively, biopolymers such as collagens, alginate, hyaluronate, and fibrin have been studied as scaffold materials for hepatocyte culture and transplantation. Among them, type I and type IV collagens have been shown to enhance the adhesion
and long-term survival of adult hepatocytes *in vitro* \(^{19-21}\). Consequently, they have been extensively applied as hepatocyte growth substrates, in the form of thin coating, soft gel, or 3D matrix \(^{14,22-24}\). It has also been reported that type I and type IV collagens enhance the proliferation and differentiation of liver stem cells and hepatic progenitors \(^{25}\).

A more complex form of natural scaffold, decellularized liver biomatrix, has also been explored. Typically, the liver tissues are subjected to a series of chemical treatment to remove the cellular components, resulting in scaffolds constituted by liver-specific extracellular matrix (ECM). Rojkind and colleagues first introduced this technique to isolate connective tissue fibers from the liver \(^{26}\). When coated onto tissue culture dishes, the biomatrix was able to improve the long-term survival and metabolic functions of adult hepatocytes \(^{26,27}\). A recent study suggested that the decellularized liver matrix could also be employed as a scaffold for long-term hepatocyte culture \(^{28}\).

Compared to their synthetic counterparts, scaffolds constructed from ECM components are more conducive to cell attachment and biological recognitions. By preserving the intact ECM of a tissue, a decellularized matrix may offer an even more “native” microenvironment as well as a growth template that captures the architecture of the tissue \(^{29}\). However, clinical application of these scaffolds is limited by batch-to-batch variation and scale-up problem in mass production \(^{28}\). On the other hand, the synthetic scaffolds can be produced in large quantity with good reproducibility. They also offer much more design flexibility: the physical, chemical, mechanical, and degradation properties of the scaffolds can be precisely controlled and tailored for specific applications \(^{30,31}\). Furthermore, cell-adhesion peptides and growth factors can be incorporated into the synthetic scaffolds to provide both spatial and temporal cues to guide tissue formation \(^{32,33}\).
Although adult hepatocyte/scaffold composites have been extensively studied for the past several decades, limited information is available regarding the cultivation of fetal liver cells on 3D scaffolds. Moreover, while the previous studies customized the culture medium for fetal liver cells, they all used scaffold materials originally designed for adult hepatocyte cultures.\textsuperscript{34-38}

This study was intended as a general survey of the currently available scaffolds regarding their effectiveness in supporting the growth and differentiation of fetal liver cells. Three representative scaffolds, decellularized liver matrix, collagen scaffold prepared from type I and IV collagens, and PLGA scaffold, were compared. The objective was to obtain information on scaffold properties that are possibly crucial to the development of fetal liver cells. First, decellularized liver matrices were produced and characterized. The three types of scaffolds were then used for fetal liver cell culture and were evaluated for their impact on cell behaviors including cell distribution, cell growth, and differentiation.

\section{3.2 MATERIALS AND METHODS}

\subsection{3.2.1 Materials}

PLGA (50:50, $M_w \sim 84,000$) was purchased from Birmingham Polymers (Pelham, AL). Chloroform was from J.T. Baker (Phillipsburg, NJ). Sodium chloride (NaCl), sodium dodecyl sulfate (SDS), and osmium tetroxide were purchased from Sigma (St. Louis, MO). Formalin (10\%, neutral, phosphate buffer) and glutaraldehyde were acquired from Polysciences (Warrington, PA). Sodium cacodylate buffer was from Electron Microscopy Sciences (Hatfield, PA). Heparine and saline were from Abbott Laboratories (North Chicago, IL). Ketamine was from Fort Dodge Animal
Health (Fort Dodge, IA) and xylazine was from Ben Venue Laboratories (Bedford, OH). Teflon vials were purchased from Cole Palmer (Vernon Hills, IL). Three-dimensional collagen matrices and nontreated 12-well plates were from BD Discovery Labware (San Jose, CA). The rest of the materials were obtained from the same sources as described in chapter 2 (section 2.2).

3.2.2 Preparation of PLGA scaffolds

PLGA scaffolds were prepared with a salt leaching technique. PLGA was dissolved in chloroform overnight to prepare a 5% (w/v) solution. Subsequently, 0.24 ml of this solution was added into a Teflon vial packed with 0.6 g of NaCl particles that were sieved to a size of 125 – 250 µm. The vials were capped, tapped gently, and allowed to sit for 30 minutes to ensure the salt was level in the polymer solution. The vials were then uncapped in the fume hood to allow the solvent to evaporate overnight. The resulting polymer-salt composites were removed from the vials, placed in tissue cassettes, and immersed in deionized water. The salt was leached out through at least 6 hourly water changes. The scaffolds were then removed from the cassettes, blotted dry, and lyophilized for 2 - 3 days. The scaffolds were stored in sealed containers with desiccant at –20ºC until use.

3.2.3 Decellularization of livers

Liver decellularization was performed on C57Bl/6 mice or Sprague-Dawley rats (Jackson Laboratory, Bar Harbor, Maine). The animals were anesthetized by intraperitoneal injection of 100/10 mg/kg body weight of ketamine/xylazine solution, respectively. Subsequently, the animal was perfused at 10 ml/min with saline that contained 1 unit/ml of Heparin using a Rabbit-Plus peristaltic pump (Rainin Instrument, Woburn, MA). The perfusion fluid was directed into the left ventricle and
drained through the hepatic vein. The animal was perfused until most of the blood was removed from the liver. The perfused liver was dissected out, and cut into pieces of ~10 X 10 X 5 mm³. The tissue chunks were incubated in sterile 1% Triton-X100 solution in a rotating shaker at 150 rpm at 37ºC. After 24 hours, the solution was switched to sterile 1% SDS and the incubation was continued. The SDS solution was changed every 2 – 3 days until the tissues became white and transparent, which took 1 – 2 weeks. Subsequently, the incubation was continued with sterile water to rinse the tissues. The water was changed every 2 - 3 days for at least 4 times. The decellularized tissues were then stored at 4ºC in sterile DPBS with 1% antibiotic-antimycotic until use.

3.2.4 Culture of fetal liver cells on matrices

The matrices were prepared for cell attachment before culture set-up. The PLGA scaffolds were soaked in 70% ethanol overnight for pre-wetting and sterilization. On day 0, collagen working solution was prepared by diluting the stock (2.9 mg/ml) 1:10 with sterile water. The ethanol-treated polymer scaffolds were rinsed twice with DPBS, coated with the collagen working solution at 37ºC for 1 hour, and rinsed with sterile water. The coated scaffolds were stored in DPBS until use. Decellularized tissues and collagen matrices were immersed in 10% FBS-containing culture medium overnight at 4ºC before use.

The isolation and processing of fetal liver cells were performed as described in Chapter 2 (section 2.2.2). The processed fetal liver cells were resuspended in incomplete DD culture medium at a concentration of 5X10⁶ cells/ml or 1X10⁷ cells/ml. Each matrix was placed in a well of a nontreated 12-well plate, and the cell suspension was added carefully in a drop-wise fashion onto each matrix. Two hundred microliters of the 5X10⁶ cells/ml cell suspension was used to seed each
PLGA scaffold. Due to their smaller sizes, collagen matrices and decellularized liver tissues were seeded with 100 µl of the 1X10^7 cells/ml cell suspension. The matrices were then placed in a cell culture incubator for 3 hours to allow cell attachment. Afterwards, incomplete DD culture medium was carefully added to each well. After 20 – 24 hours of culture, the culture supernatant was removed and the matrices were rinsed with DPBS to remove non-adherent cells. The rinsed matrices were transferred into a new nontreated 12-well plate, and cultured with complete DD medium. Culture medium was changed every 2 – 3 days thereafter.

3.2.5 Assessment of cell growth

Cell growth on the matrices was monitored by MTS assay and DNA assay. To perform MTS assay, culture medium was removed and the matrices were rinsed with DPBS to remove residual medium with phenol red. The matrices were then transferred to a new 12-well plate to exclude cells growing off the matrices. The MTS assay was then performed as described in Chapter 2 (section 2.2.4). Again, MTS assay was performed on day 0 on a serial titration of the processed fetal liver cells (8X10^6 – 7.8X10^3 cells/well at 1:2 titration) to test the linearity of the assay.

Following the MTS assay, the supernatant was aspirated from the culture wells, which were then rinsed with DPBS. Each matrix was then transferred to an Eppendorf tube containing 200 µl of DPBS, and stored at –80°C until use. To extract DNA, the samples were thawed, mixed with 200 – 400 µl of lysis solution, and cut into fine pieces with clean scissors. The samples were sonicated as described in section 2.2.4, and stored at -80°C until use. Before DNA assay, the sonicated samples were thawed and centrifuged in a microcentrifuge at 14,000 rpm at 4°C for 30 minutes to spin down all debris. The supernatant was transferred to a new tube and quantified for DNA as described in section 2.2.4. Standard curves using serially diluted day 0
fetal liver cells ($8 \times 10^6$ – $7.8 \times 10^3$ cells at 1:2 titration) were used to test the linear ranges of the assay at various sample dilutions.

3.2.6 Analysis of hepatocyte functions

Albumin secretion was analyzed as described in Chapter 2 (section 2.2.5).

3.2.7 Histology and PAS staining

Decellularized tissues and cell-seeded matrices were rinsed with DPBS, and fixed with 10% neutral formalin at RT for 4 hours. The fixed samples were transferred into 70% ethanol and submitted to Research Histology at Yale University for paraffin embedding. Hematoxylin-Eosin (H&E) and PAS stainings were performed by Research Histology on 5 µm thick paraffin sections.

3.2.8 Scanning electron microscopy (SEM)

Decellularized tissues and cell-seeded scaffolds were fixed, dehydrated, and dried before SEM examination. Briefly, the samples were rinsed with 0.1 M sodium cacodylate buffer, and fixed with 2.5% glutaraldehyde (in 0.1M sodium cacodylate buffer) overnight. The fixed samples were rinsed 3 times with the cacodylate buffer: the first and last rinses at RT on a shaker for 15 minutes, the second one at 4°C for overnight. The samples were post-fixed in 1% osmium tetroxide (in 0.1M sodium cacodylate buffer) at 4°C for 1 hour. Following a quick water rinse, the samples were serially dehydrated with 70%, 85%, 95%, 100%, and 100% ethanol, each for 10 minutes on a shaker. The samples were then dried using a critical point dryer (Polaron, Watford, England).

All samples to be examined for SEM were coated with gold using a 108 auto sputter coater (Cressington Scientific Instruments, Valencia, PA). The samples were
examined with a Philips XL series SEM, at 5 kV of acceleration voltage, spot 3 of beam size, and a working distance around 10 mm.

3.3 RESULTS

3.3.1 Characterization of decellularized liver matrices

The decellularization procedure produced white, sponge-like tissues (Figure 3.1). The procedure also led to a significant reduction in tissue volume, presumably due to the removal of cellular contents.

H&E staining showed that the decellularized matrices were devoid of cytoplasm or nuclei staining, indicating a complete removal of the cellular components (Figure 3.2). Correspondingly, no MTS activity or DNA content was detected with the decellularized tissues (data not shown).

On the other hand, the liver tissue structure and vascular network appeared to be at least partially preserved. The removal of liver cells left behind a mesh formed by fiber-like structures. In addition, many central veins and portal triads were discernable in the decellularized matrices (Figure 3.2). However, these structures were much closer to each other than in normal livers, further demonstrating the tissue shrinkage after removal of liver cells.

SEM showed that the decellularized matrices were highly porous and non-homogenous (Figure 3.3). Two types of structures were often observed: networks of fibers forming pores of varied sizes, and coral-like features with small (less than 5 µm) but more uniform pores.
Figure 3.1 Appearance of liver tissues before (A) and after (B) decellularization. The tissues were placed in a 35 mm petri dish.
Figure 3.2  H&E staining of liver tissues before (A) and after (B) decellularization. Structures of central veins (CV) and portal triads (PT) were preserved. Magnification = 10X. Scale bar = 500 µm.
Figure 3.3 SEM of decellularized liver matrices. The matrices were porous and non-homogenous (A), containing networks of fibers forming pores of varied sizes (B) and coral-like features with small but more uniform pores (C). Scale bar = 50 µm (A) or 10 µm (B – C).
3.3.2 Fetal liver cell culture on scaffolds

Experiments in this section compared the growth and differentiation of E14.5 mouse fetal liver cells cultured on decellularized liver matrices, collagen scaffolds, and PLGA scaffolds. As mentioned earlier, the structure of decellularized matrices was heterogeneous. In contrast, collagen matrices and PLGA scaffolds possessed more defined porous structures, with pore size at 100 – 200 µm (provided by product manufacturers) and 125 – 250 µm, respectively (Figure 3.4).

Cell distribution on scaffolds

After two weeks of culture, substantially more cells were detected on PLGA scaffolds than on collagen or decellularized matrices (Figure 3.5). In all cases, the majority of the cells were distributed on the surfaces of the scaffolds, forming one or more cell layers. On decellularized matrices, the cells that did penetrate the matrices did not seem to have preferred attachment sites: they adhered to the filamentous networks as well as to the lumen of vascular structures.

SEM showed that, in two weeks, cell layers completely covered the surfaces of PLGA scaffolds and partially covered those of collagen scaffolds. Cells in these layers appeared to be relatively flat and displayed the characteristic polyhedral shape of hepatocytes (Figure 3.6). Cells on the decellularized matrices were more scattered, and appeared to be more heterogeneous: both flat, spread-out and round, compact cells with the polyhedral shape were observed.

Cell growth

Both DNA assay and MTS assay showed that the fetal liver cells continued to proliferate over two weeks on all three matrices (Figure 3.7). However, cell growth was more rapid on PLGA scaffolds. At the end of a 13-day culture period, the DNA
Figure 3.4  Structures of decellularized liver matrices (A), collagen matrices (B), and PLGA scaffolds (C), imaged by SEM. Scale bar = 50 µm (A), 100 µm (B), or 200 µm (C).

5 Figure 3.4A is the same as Figure 3.3A.
Figure 3.5  H&E staining of E14.5 fetal liver cells cultured for 2 weeks on
decellularized liver matrices (A), collagen matrices (B), and PLGA scaffolds (C). The
cells are identified by blue-black staining of the nuclei and pink staining of the
cytoplasm. Magnification = 20X. Scale bar = 200 µm.
Figure 3.6  SEM of E14.5 fetal liver cells cultured for 2 weeks on the scaffolds. Cells on PLGA (A) and collagen (B) scaffolds appeared as layers of flat hepatocytes, whereas cells on decellularized liver matrices were a mix of flat (C) and three-dimensional hepatocytes (D). Scale bar = 20 µm (A – B, D) or 10 µm (C).
content of cultures on PLGA scaffolds was 1.3 and 4.8 times of those on collagen and decellularized matrices, respectively.

Albumin synthesis

The total albumin secretion rate increased rapidly during the first week in all cultures (Figure 3.8A). Subsequently, the secretion level of cultures maintained on PLGA scaffolds continued to increase at a slower rate. In contrast, the total secretion rate declined and stabilized at a lower level in the cultures on collagen and decellularized matrices.

In cultures maintained on decellularized liver matrices or PLGA scaffolds, the specific albumin secretion rate increased during the first 7 days and stabilized at the peak level afterwards (Figure 3.8B). The specific secretion rate of cultures on collagen scaffolds remained constant during the first week and declined after that. Opposite to what was observed with cell growth, the specific secretion rate of cultures on decellularized matrices at day 7 was 2.5 - 2.6 times of those on collagen and PLGA scaffolds. At day 13, it was 4.2 and 2.3 fold of those on collagen and PLGA scaffolds, respectively.

Glycogen accumulation

Glycogenesis was detected in all cultures, as demonstrated by the presence of cells stained for PAS (Figure 3.9). Quantitative analysis needs to be performed in the future to compare the extent of glycogenesis on the three scaffolds.
Figure 3.7 Proliferation of E14.5 mouse fetal liver cells as measured by DNA assay (A) and MTS assay (B) when cultured on decellularized liver matrices (DCL), collagen matrices (CO), and PLGA scaffolds (PLGA).
Figure 3.8  Albumin secretion of E14.5 mouse fetal liver cells cultured on decellularized liver matrices (DCL), collagen matrices (CO), and PLGA scaffolds (PLGA), as indicated by the total secretion rate (µg albumin/well-day, A) and the specific secretion rate (µg albumin/µg DNA-day, B).
Figure 3.9 Glycogenesis of E14.5 mouse fetal liver cells cultured for 2 weeks on decellularized liver matrices (A), collagen scaffolds (B), and PLGA scaffolds (C). Magnification = 40X. Scale bar = 100 μm.
3.4 DISCUSSION

3.4.1 Decellularized liver matrices

Results from this study demonstrated that the decellularization method described here can efficiently remove the cellular components of liver tissues. As has been observed with other decellularization procedures, removal of the liver cells leaves behind a three-dimensional, porous matrix. However, this study is among the first to characterize the structures of the matrix in detail. Histology analysis showed that the matrix is an intricate network formed by fiber-like, non-cellular elements. Further analysis is required to determine whether these elements are the connective tissue fibers of the liver, the capillary network, or a combination of both. However, it is clear that larger anatomical structures, such as the central veins and portal triads, are preserved. Furthermore, SEM imaging showed that the observed network of fibers is not homogeneous, and contains at least two types of structures: networks of fibers forming pores with varied sizes, and coral-like features with small but uniform pores. Further analysis is required to determine the origin of these structures.

Cell culture experiments showed that mouse fetal liver cells can attach and proliferate on the decellularized liver matrices. However, cell growth was much slower than on collagen matrices and PLGA scaffolds. Previous studies also showed that adult hepatocytes cultured on decellularized liver matrices possess lower growth capacity than under other conditions (e.g. collagen sandwich). The observed variation in cell proliferation could be a result of the different chemical and physical properties of the scaffolds. In addition, the slow cell growth on decellularized liver matrices may be partially due to inefficient cell attachment, possibly caused by the denaturation or removal of binding sites in the matrices by the
decellularization procedure. It is also possible that, even with the extensive washings performed here, the decellularized matrices still contained residual detergents that inhibited cell growth. Further optimization of the decellularization method is required to address these issues.

The liver tissue structure preserved in the decellularized matrices did not seem to guide cell organization, as the cells did not show preference in attachment sites. This is consistent with a previous report that hepatocytes do attach to biomatrices derived from other organs such as the aorta and mammary glands, although the cells express less differentiated functions than on the liver biomatrix\textsuperscript{40}. Therefore, to take advantage of the architectural template provided by a decellularized matrix, alternative seeding methods would be required to restrict the fetal liver cells away from the lumen of blood vessels\textsuperscript{39}.

3.4.2 Differential cellular responses to the scaffolds

As described in Chapter 2, when fetal liver cells are cultured in medium DD on a 2D surface, albumin synthesis increases along with DNA content, presumably due to the continual generation, proliferation, and differentiation of hepatocyte precursors. However, this was not the case when the cells were cultured on the 3D matrices tested in this study. The decellularized matrices, which did not support efficient cell growth, led to higher specific albumin secretion rate than the other two scaffolds (Figures 3.7 and 3.8). These results suggested that, in addition to the soluble signals provided in the medium, the culture substrata also play an important role in regulating the growth and differentiation of fetal liver cells and can even alter their responses to soluble signals.

Although the three types of matrices tested in this study cannot be compared systematically due to their drastically different compositions and architectures, a
qualitative inspection of their properties still yields clues to how the scaffolds may affect the fetal liver cells. First, significant morphological changes were observed with the PLGA scaffold during the experiments. At the end of a 2-week culture period, the PLGA scaffold appeared to be thinner and its diameter decreased by almost 50%. In addition, the scaffold became irregular in shape. In contrast, no noticeable changes were observed with the decellularized and collagen matrices. These observations implied that the three types of matrices may differ in their degradation rates. Second, although the mechanical properties of the scaffolds were not quantified, the PLGA and collagen scaffolds appeared to be much more rigid than the decellularized matrix. Taken together, it appeared that, in addition to its chemical composition and surface chemistry, the mechanical and degradation properties of a 3D scaffold might also contribute to regulating the proliferation and maturation of fetal liver cells. Since these properties are easier to adjust in synthetic scaffolds than in decellularized and collagen matrices, it would be interesting to see if the fetal liver cells are indeed affected when the scaffold properties are altered.

In summary, the three types of scaffolds tested in this study did not appear to be ideal for fetal liver cell culture, as none of them could support both efficient growth and differentiation. The results also hinted at the importance of the scaffold properties. Further investigations using synthetic scaffolds will help to elucidate their effects and to define the optimal culture substrates for fetal liver cells, which can potentially incorporate the desirable properties of both natural and synthetic materials.
BIBLIOGRAPHY


CHAPTER 4
EFFECTS OF SCAFFOLD PROPERTIES ON THE DEVELOPMENT OF FETAL LIVER CELLS

4.1 INTRODUCTION

In the past several decades, much effort has been directed towards designing scaffolds with optimal properties for liver tissue engineering. In particular, many studies have focused on tailoring the surface characteristics of the materials to control their interactions with hepatocytes \(^{1-3}\). On the other hand, the bulk properties such as mechanical strength and degradation behaviors are primarily designed to provide the required growth space and mechanical support during tissue generation \(^{4,5}\). However, numerous studies have shown that these properties also play an important role in regulating the morphology, aggregation, growth, and function of adult hepatocytes \(^{6-8}\), and therefore should be taken into consideration to control the cell-matrix interactions.

As for fetal liver cells, research regarding their tissue engineering applications has been limited. Moreover, in the studies reported so far, although biochemical signals such as OSM were supplemented to suit the developmental needs of fetal liver cells, the scaffold materials were adopted from adult hepatocyte cultures without modifications \(^{9-13}\). As demonstrated in Chapter 3, the culture substrates do influence the behaviors of fetal liver cells and can even alter their responses to soluble signals. Therefore, it is vital to characterize the substrate-induced responses of fetal liver cells and tailor the material properties to achieve the desired cell behavior.

Similar to observations with adult hepatocytes, results from Chapter 3 implied that the mechanical and degradation properties of a 3D scaffold might also contribute to its regulation of fetal liver cells. The study presented in this chapter would attempt
to investigate this issue in further details using PLGA (50:50), polycaprolactone (PCL), and their blends at various ratios. Like PGA and PLA, PCL is a biodegradable polyester due to the susceptibility of its aliphatic ester bonds to hydrolysis. However, it degrades at a considerably slower rate and exhibits more elasticity than its poly(α-hydroxy acids) counterparts. Due to these characteristics, PCL has been combined with other polymers in the form of copolymers or blends for various applications 14-17. In this study, PLGA, PCL and their blends served as model systems to evaluate the effects of scaffold properties on fetal liver cells. First, the properties of the materials were characterized. Scaffolds prepared from these polymers were then used for fetal liver cell culture and were evaluated for their impact on cell behaviors including cell distribution, cell growth, and differentiation.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of polymer scaffolds

Polymer scaffolds were prepared from PLGA (50:50, $M_w \sim 84,000$, Birmingham Polymers, Pelham, AL), PCL (M$_w$ $\sim 65,000$, M$_n$ $\sim 42,500$, Sigma, St. Louis, MO), or PLGA/PCL blends using the salt leaching technique. PLGA and PCL were dissolved in chloroform overnight to prepare 5% (w/v) solutions. The solutions were mixed at varied ratios to prepare PLGA/PCL (75/25), PLGA/PCL (50/50), and PLGA/PCL (25/75) blends. Scaffolds were prepared from these polymer solutions as described in Chapter 3 (section 3.2.2), except that the size of the NaCl particles used in this study was 250 - 425 μm and 0.4 g of NaCl was used per scaffold.
4.2.2 Test of degradation rate

The polymer scaffolds were pre-wet with 70% ethanol and coated with collagen as described in Chapter 3 (section 3.2.4). The coated scaffolds were rinsed twice with deionized water, blotted dry, and lyophilized. The weight and dimensions (diameter and thickness) of the dried scaffolds were recorded. Each scaffold was then placed in 1 ml/well of DPBS + 1% antibiotic-antimycotic in a nontreated 12-well plate, and incubated in a cell culture incubator. The solution was changed every 2 – 3 days. At designated time points, the scaffolds were removed from the plate, rinsed twice with deionized water, blotted dry, and lyophilized. The dimension and weight of these scaffolds were measured and compared with the initial values.

4.2.3 Test of hydrophilicity

The hydrophilicity of the scaffolds was evaluated in two tests. In the first test, the ability of water to wet the scaffolds was determined. The dry scaffolds were weighed, and placed in a bacterial grade petri dish. One hundred thirty microliters of water was added to the surface of the scaffolds in a drop-wise fashion. After 10 minutes to allow the water to absorb, the scaffolds were held vertically and shaken to remove the unabsorbed water. The scaffolds were weighed again to determine the amount of water that was absorbed.

In the second test, the contact angles of the polymers with water were measured. Polymer films were used instead of the 3D scaffolds to simplify the measurement. Polymer solutions, prepared as described in section 4.2.2, were added to a glass slide at 0.1 – 0.2 ml/slide. The solution was spread over the slide using a glass Pasteur pipette to form a thin polymer layer. While the layer was still tacky, another 0.2 – 0.3 ml of solution was spread onto the slide to ensure that the polymer film completely covered the slide. The slides were left in fume hood overnight to
allow the solvent to evaporate. Subsequently, some of the slides were pre-wet with 70% ethanol and coated with collagen as described in Chapter 3 (section 3.2.4). Both the uncoated and collagen-coated slides were lyophilized before contact angle measurement. The contact angle measurement was performed using a goniometer (Rame-Hart, Mountain Lakes, NJ), with a water droplet size of 2 mm.

4.2.4 Culture and characterization of fetal liver cells on scaffolds

Fetal liver cells were isolated and processed as described in Chapter 2 (section 2.2.2). The polymer scaffolds were pretreated for cell culture as described in Chapter 3 (section 3.2.4). The processed fetal liver cells were resuspended in incomplete DD culture medium at a concentration of 5X10^6 cells/ml. Each scaffold was seeded with 200 µl of the cell suspension and cultured as described in section 3.2.4. Characterization of the cultures, including cell growth, hepatocyte functions, histology, PAS staining, as well as SEM imaging, were all performed as described in Chapter 3 (section 3.2.5 – 3.2.8).

4.3 RESULTS

4.3.1 Characterization of PLGA/PCL scaffolds

Scaffold morphology

Scaffolds prepared from PLGA, PCL, and their various blends all showed similar architecture (Figure 4.1). All scaffolds exhibited high porosity, with rectangular pores mostly 200 - 400 µm in size.
Figure 4.1  SEM of scaffolds prepared from PLGA(A), PLGA/PCL (75/25) (B), PLGA/PCL (50/50) (C), PLGA/PCL(25/75) (D), and PCL (E). Scale bar = 200 µm.
**Hydrophilicity**

PLGA, PCL, and their blends were all relatively hydrophobic, as indicated by their high contact angles with water (Figure 4.2A). PLGA had the lowest contact angle among all at 73.5 ± 1.4°. When it was blended with PCL, the contact angle increased as the percentage of PCL (%PCL) increased from 0% to 50%. PLGA/PCL blends with 50% or more PCL had similar contact angles as pure PCL.

Collagen coating rendered the polymers more hydrophilic, as demonstrated by the decreases in their contact angles (Figure 4.2A). However, the reductions were less dramatic than what was previously reported\(^3\), probably due to differences in collagen concentration and coating method. Nevertheless, the coating seemed to mask the hydrophilicity variations observed with the uncoated polymers, as the contact angles of the coated materials were not significantly different.

Since the polymers were utilized in the form of porous scaffolds for fetal liver cell cultures, the hydrophilicity of the scaffolds was tested by their ability to absorb water (Figure 4.2B). The collagen-coated scaffolds were able to absorb water of 3.6 – 4.6 times of their weight. No statistically significant difference was noted among the samples.

**Degradation**

When incubated in DPBS, the PLGA scaffold exhibited the most rapid reductions in mass, diameter, and thickness among all samples (Figure 4.3). Blending PLGA with PCL slowed down the decrease of mass and dimension, with the rate of decrease inversely correlated with %PCL.

For scaffolds with more than 50% PLGA, their dimensions decreased far more dramatically than their masses. For example, the PLGA scaffold lost 4% of its mass after 3 weeks, whereas its diameter and thickness were reduced by 53% and 26%,
Figure 4.2 Hydrophilicity of polymers with varied PLGA/PCL content, as assessed by water contact angle analysis of the polymer films before and after collagen coating (A), and water absorption of collagen-coated scaffolds (B). “*” indicates $p < 0.05$ compared with PLGA/PCL(100/0).
Figure 4.3  Degradation of scaffolds with varied PLGA/PCL content, as assessed by changes in mass ("W", A), dimension ("D", B), and thickness ("T", C). The values at each time point (W_t, D_t, T_t) were normalized based on the initial values (W_0, D_0, T_0).
respectively. The masses and dimensions of PLGA/PCL (25/75) and PCL scaffolds remained almost unchanged over 3 weeks.

4.3.2 Responses of the fetal liver cells to PLGA/PCL scaffolds

Scaffold appearances and cell distribution

The morphology of scaffolds seeded with fetal liver cells changed in a similar way as that of their unseeded counterparts (Figure 4.4). After 3 weeks of culture, increasing level of dimension reduction was observed with higher percentage of PLGA (%PLGA). The PLGA and PLGA/PCL (75/25) scaffolds also became irregular in shape. The appearance of PLGA/PCL (25/75) and PCL scaffolds did not change noticeably. In addition, as the culture proceeded, increasing amount of ECM fibers was observed projecting from all the scaffolds (Figure 4.5).

H&E staining showed that, when cultured on the PLGA scaffold, fetal liver cells penetrated the scaffold during the first 2 weeks but retreated mostly to the scaffold surface by day 21 (Figure 4.6). On scaffolds prepared from PLGA/PCL blends or PCL, however, the cells were observed penetrating the scaffolds and lining the pores throughout the culture period. This observation was confirmed by SEM analysis (Figure 4.7). By day 7 of culture, the fetal liver cells had formed a confluent layer that covered the surface of the PLGA scaffold, rendering its pore structure largely invisible. In contrast, the other scaffolds remained porous throughout the 21-day culture period, with the cells residing both outside and within the pores, forming cell layers and aggregates. On all scaffolds, many cells were found displaying the polyhedral shape characteristic of hepatocytes. The cell size of these presumed hepatocytes ranged from < 10 µm to 40 µm, indicating their varied differentiated
Figure 4.4 Appearance of PLGA/PCL scaffolds seeded with E14.5 mouse fetal liver cells after 3 weeks of culture. The PLGA/PCL content of the scaffolds ranged from 100/0 (A), 75/25 (B), 50/50 (C), 25/75 (D), to 0/100 (E). The brown color seen in some scaffolds was due to residuals of the colored compound formed during MTS assay.
Figure 4.5  ECM fibers extended out from all PLGA/PCL scaffolds seeded with E14.5 mouse fetal liver cells. Representative images were shown here (day-14 cultures). Magnification = 4X (A) or 40X (B).
Figure 4.6 H&E staining of E14.5 fetal liver cells cultured for 15 days (A1) and 21 days (A2) on PLGA scaffolds, or for 15 days on scaffolds with PLGA/PCL content of 75/25 (B), 50/50 (C), 25/75 (D), and 0/100 (E). Magnification = 10X. Scale bar = 500 µm.
Figure 4.7  SEM of E14.5 fetal liver cells cultured for 21 days on scaffolds with PLGA/PCL content of 100/0 (A), 75/25 (B), 50/50 (C), 25/75 (D), and 0/100 (E). Hepatocytes on the scaffolds appeared to be either flat (F) or three-dimensional (G) with cell size ranging from < 10 μm to 40 μm. Local deformation of the scaffold could be observed at scaffold-cell contact points (H – I). Scale bar = 100 μm (A – E), 10 μm (F), or 5 μm (G - I).
Figure 4.7 (Continued).
states. At high magnification, local deformation of the scaffold caused by the cells could be observed.

**Cell growth**

All scaffolds supported continuous cell growth during the first week of culture (Figure 4.8). Subsequently, the total DNA content reached a plateau on PLGA, PLGA/PCL (25/75), and PCL, but continued to increase for another week on PLGA/PCL (75/25) and PLGA/PCL (50/50). At the end of a 21-day culture, cell expansion (based on the fold of increase in DNA content) on PLGA/PCL (75/25) and PLGA/PCL (50/50) scaffolds was 1.6 - 2.5 times of that on the others.

During the first week, cell growth appeared to be inversely correlated with the %PCL of the scaffolds: cellular DNA content was the highest on PLGA scaffolds and decreased linearly as the scaffolds contained increasing amount of PCL (Figure 4.8A). The growth response became parabolic thereafter: DNA content and MTS activity were the highest on scaffolds with 25% - 50% PCL, but decreased when the %PCL was outside of this range.

**Albumin synthesis**

On all scaffolds, the total albumin secretion rate peaked at day 8 – 10. Afterwards, the secretion rate was stabilized at a lower level on scaffolds with 50% or more PCL (Figure 4.9A). In contrast, the secretion level decreased continuously on PLGA and PLGA/PCL (75/25). The change of total secretion level versus PLGA/PCL content showed a similar trend as cell growth: during the first week, the secretion level was the highest on PLGA scaffolds, and decreased in a linear manner as the scaffolds contained more PCL. In the following 2 weeks, however, a parabolic response to
Figure 4.8  Proliferation of E14.5 mouse fetal liver cells as measured by DNA assay (A) and MTS assay (B) when cultured on scaffolds with varied PLGA/PCL content. “*” and “+” indicate $p < 0.05$ compared with cultures on PLGA/PCL(100/0) and PLGA/PCL(75/25) scaffolds, respectively.
Figure 4.9  Albumin secretion of E14.5 mouse fetal liver cells when cultured on scaffolds with varied PLGA/PCL content, as indicated by the total secretion rate (µg albumin/well-day, A) and the specific secretion rate (µg albumin/µg DNA-day, B). “*” and “+” indicate $p < 0.05$ compared with cultures on PLGA/PCL(100/0) and PLGA/PCL(0/100) scaffolds, respectively.
%PCL was again observed. Blending PLGA with 25% - 50% PCL led to the highest secretion level, further increase or decrease of %PCL both led to reduced secretion.

On the other hand, the addition of PCL appeared to have a favorable effect on the specific albumin secretion rate (Figure 4.9B). Although the specific secretion rate was similar on all scaffolds during the first week, it increased almost linearly with %PCL thereafter. Furthermore, the specific secretion rate declined after about 10 days on scaffolds with 50% or less PCL, but was stabilized on PLGA/PCL (25/75) and PCL.

**Glycogen accumulation**

Glycogen-accumulating cells were detected in all cultures, as demonstrated by PAS staining (Figure 4.10). The percentage of stained cells increased over time on all scaffolds. Quantitative analysis needs to be performed in the future to determine the effect of PLGA/PCL content on glycogenesis.

### 4.4 DISCUSSION

This study employed blends of PLGA/PCL as a model system to examine the influences of scaffold properties on fetal liver cells. Variation of the scaffold’s relative content of PLGA and PCL induced differential cellular responses in a time-dependent manner. Initially, cell proliferation was more rapid in the presence of PLGA but down-regulated by PCL. However, as the culture proceeded, maximal cell growth was obtained on scaffolds containing intermediate amount (25% - 50%) of PCL. On the other hand, the specific albumin secretion rate was enhanced with increasing %PCL.
Figure 4.10 Glycogenesis of E14.5 fetal liver cells cultured for 2 weeks on scaffolds with PLGA/PCL content of 100/0 (A), 75/25 (B), 50/50 (C), 25/75 (D), and 0/100 (E). Magnification = 40X. Scale bar = 100 µm.
It has been suggested that cell adhesion strongly depends on the hydrophilicity of the substratum.\(^{18-20}\) In this study, contact angle analysis indicated that PCL was slightly more hydrophobic than PLGA (50:50). Consequently, the PLGA/PCL blends were less hydrophilic than PLGA. However, the variation in hydrophilicity was not likely the primary cause of the observed cell behavior: first, scaffolds containing 50% or more PCL exhibited similar contact angles as pure PCL, but they led to significantly different growth and differentiation kinetics. In addition, all the polymers possessed similar wettability after collagen coating, as attested by both contact angle analysis and water absorption test. Furthermore, past experiments showed that fetal liver cells synthesized large amount of ECM when cultured on 2D surfaces (data not shown). In 3D culture experiments, all scaffolds were found to project increasing amount of ECM fibers as the culture proceeded (Figure 4.5). Therefore, it is possible that the fetal liver cells deposited their own ECM on the scaffolds, further masking the hydrophilicity variations in long-term culture.

When incubated in DPBS for 3 weeks, the PLGA scaffold lost only 4% of its mass, but its diameter and thickness were reduced by 53% and 26%, respectively. It is known that aliphatic polyesters undergo bulk degradation. In this process, cleavage of the polymer backbone, which leads to decreases of molecular weight (MW) and mechanical strength, precedes mass loss.\(^{21}\) PLGA(50:50) has a degradation time of 1 - 2 months (provided by the product manufacturer). The PLGA scaffold used in this study may degrade even faster, as its highly porous structure allows water access to the scaffold’s interior. Hence, the PLGA scaffold’s rapid reduction in dimension could be caused by the degradation-associated loss of mechanical strength, which led to a collapse of the pore structures. This postulation is consistent with the observation that PLGA scaffolds incubated in DPBS for more than 1 week became more fragile
and broke more easily during handling. However, it needs to be confirmed by analysis of the hydrolyzed scaffolds for changes in MW and mechanical properties.

Consistent with previous reports\textsuperscript{22, 23}, the mass loss of PCL was much slower than that of PLGA. Not surprisingly, blends of PLGA and PCL demonstrated erosion properties that varied between these two extremes, with less mass loss at increased \%PCL. More importantly, incorporation of the slow-degrading PCL alleviated the reduction of the scaffold’s dimension, possibly by preventing the rapid loss of mechanical strength that preceded the mass loss. At present, it is not known whether the observed phenomenon was contributed solely by PCL, or in conjunction with a slower degradation of PLGA in the presence of PCL. The PLGA (50:50) utilized in this study has a Young’s modulus of 1 – 3 $\times$ 10\textsuperscript{6} kPa, whereas the modulus of PCL is an order of magnitude lower at 2 – 3 $\times$ 10\textsuperscript{5} kPa (provided by product manufacturers). Although the mechanical properties of the scaffolds are yet to be quantified, it did appear that the materials became more pliable with increasing \%PCL and decreasing \%PLGA. Hence, it is plausible that blending PLGA and PCL at different ratios created a series of substrates with systematically varied mechanical and degradation properties.

Based on the above analysis, it is possible that the mechanical and degradation characteristics of the scaffolds regulated the growth and differentiation of fetal liver cells in multiple ways. In the early stage of the culture, scaffolds with higher \%PLGA and presumably higher Young’s modulus supported better cell growth. This is similar to previous observations that rigid substrates promote the proliferation of fibroblasts and preosteoblasts\textsuperscript{24, 25}. As the culture proceeded, the degradation-associated loss of mechanical strength, combined with the traction forces exerted by the cells\textsuperscript{26-31}, caused a collapse of the pore structures in scaffolds prepared from PLGA. This likely led the cells to spread on the scaffold surface, sealing off the pores and cutting off
nutrient supply to the cells that might have grown into the pores earlier. The addition of PCL reduced the loss of mechanical strength and thus preserved the pore structures, which benefited cell expansion by enhancing mass transport and providing increased growth area. Consequently, scaffolds with higher stiffness but remained porous, such as PLGA/PCL (75/25) and PLGA/PCL (50/50), promoted the best cell growth over the 3-week culture period. On the other hand, increasing the %PCL of the scaffold, which presumably decreased its Young’s modulus, promoted the albumin secretion of fetal liver cells in an almost linear fashion. This is consistent with previous studies on a wide range of cell types, which showed that the expression of differentiated functions is favored on more compliant substrates.

Increasing evidences suggest that anchorage-dependent cells respond to their substrate via an “inside → outside → in” feedback loop that couples the cellular responses to the stiffness of the matrix: as the cells adhere, part of the contractile forces generated by the actin/myosin microfilaments are transmitted to the substrate through adhesion complexes (referred to as traction forces), causing local deformation of the substrate depending on its stiffness. In turn, the cells sense the level of matrix resistance and respond by adjusting cellular responses such as adhesion, morphology, migration, growth, and differentiation. For differentiated cell types such as endothelial cells, it has been suggested that substrates with high resistance elevate cell traction forces and promote cytoskeleton reorganization that may result in large-scale cell shape changes. These events drive the progression through the G\textsubscript{i}/S restriction point and entry into the S phase of the cell cycle. The cell cycle progression also increases the cell’s responsiveness to soluble mitogens, resulting in enhanced cell proliferation. In contrast, softer substrates do not promote this series of actions and hence lead to differentiation and enhanced tissue-specific functions. Findings
from this and other studies\textsuperscript{25, 37} suggest that the proposed mechanism may also extend to less mature cells such as fetal cells and progenitor cells.

In summary, the PLGA/PCL model system provided insights on the important role of 3D scaffolds in regulating the development of fetal liver cells. The findings suggested that cell expansion requires rigid, porous scaffolds with appropriate degradation rate, while differentiation is enhanced on more pliable substrates. In the future, it would be worthwhile to design test systems with decoupled mechanical and degradation properties so that the individual effects of these parameters could be examined. Findings from these studies will not only help to design optimal culture systems for fetal liver cells, but may also provide useful information for applications of other stem cells and progenitor cells.

Finally, this study illustrated the feasibility of pairing a fast-degrading, rigid material and a slow-degrading, pliable material to support optimal growth and differentiation. For example, PLGA/PCL (50/50) supported efficient cell proliferation (Figure 4.8), whereas PLGA/PCL (25/75) led to high albumin synthesis (Figure 4.9). By further fine-tuning the PLGA-to-PCL ratio, it may be possible to create a scaffold that would initially provide a rigid substrate that encourages cell expansion while maintaining its structural integrity. In later stages, the scaffold would become more pliable and hence promote maturation of the expanded cells. This approach may be especially beneficial to applications involving stem cells and progenitor cells, given their inherently high capacity to proliferate and differentiate.
BIBLIOGRAPHY


Fetal liver cells, which contain a high percentage of liver progenitors, possess high growth potential and can readily repopulate the liver. Hence, they may provide an alternative cell source for liver tissue engineering. In order to generate sufficient number of functional hepatocytes from fetal liver cells for clinical applications, it is necessary to establish a culture system that supports their optimal expansion and maturation. This thesis explored the influences of several culture variables in order to identify properties that lead to control of the proliferation and differentiation of fetal liver cells. Specifically, the effects of soluble signals and 3D scaffold properties were examined.

The effects of biochemical signals were studied in Chapter 2. A comparison of the representative medium formulations used in current research showed that, with regard to their intended usage, these media are not as effective as previously reported. Subsequently, the study demonstrated that the growth and differentiation kinetics of fetal liver cells are influenced not only by the regulatory factors that are present, but also by the timing of their addition. HGF is required throughout the culture to induce the differentiation of ALB− stem cells into hepatic precursors and to ensure the continual proliferation of stem cells and precursors. The constant presence of protective agents such as ascorbic acid helps to enhance cell survival, resulting in improved cell expansion and hepatocyte functions. Finally, timing of the addition of maturation factors such as OSM determines the kinetics of cell differentiation. In the presence of HGF, addition of OSM from the very beginning results in concurrent growth and differentiation. Based on these findings, a modified culture medium was
developed to achieve both optimal growth and differentiation. In average, cell expansion in this medium was about 2 fold of that under other conditions. At the end of a 2-week culture period, the total albumin secretion rate and the specific albumin secretion rate in medium DD were 2 - 4 and 2 - 3 times of the maximal values obtained with other conditions, respectively.

In Chapter 3, a general survey of the 3D scaffolds used in liver tissue engineering was conducted regarding their effectiveness in supporting fetal liver cell cultures. Three representative scaffolds, decellularized liver matrix, collagen scaffold, and PLGA scaffold, were compared. The results showed that these scaffolds did not appear to be ideal for fetal liver cell cultures, as none of them could support both efficient growth and differentiation. More importantly, the study demonstrated that the culture substrata play an important role in regulating the behavior of fetal liver cells and can even alter their responses to soluble signals. A qualitative inspection of the degradation and mechanical characteristics of the matrices further implied that these properties, in addition to the scaffold’s chemical composition and surface chemistry, might contribute to regulating the proliferation and maturation of fetal liver cells.

Chapter 4 investigated the impact of scaffold properties in more details using PLGA, PCL, and their blends as a model system. The findings suggested that, for fetal liver cells, cell expansion requires rigid, porous scaffolds with appropriate degradation rate, while differentiation is enhanced on more pliable substrates. In further studies, test systems with decoupled mechanical and degradation properties are required to determine the individual effects and optimal values of these scaffold parameters. This study also illustrated the feasibility of pairing a fast-degrading, rigid material and a slow-degrading, pliable material to support optimal growth and differentiation. A scaffold thus created would initially provide a rigid substrate that encourages cell
expansion while maintaining its structural integrity. In later stages, the scaffold would become more pliable and hence promote maturation of the expanded cells.

The *in vitro* culture of fetal liver cells is a complex and dynamic process due to 1) the presence of multiple cell populations at varied developmental stages, and 2) concomitant proliferation and multi-step differentiation of these populations. Overall, studies presented in this thesis suggested that the behavior of fetal liver cells is regulated by both the chemical and physical cues present in their microenvironment. Furthermore, they are influenced not only by what regulatory signals are present, but also by when the signals are present. Accordingly, an ideal culture environment should be a dynamic integration of biochemical signals and substrate-related signals to control the growth and differentiation kinetics of fetal liver cells. This may also be true for other stem cells and progenitor cells with inherently high capacity to proliferate and differentiate.